DIETARY AMINO ACID REQUIREMENTS OF THE ALMOND MOTH, CADRA CAUTELLA (WALKER), BASED ON RADIOMETRIC AND CARCASS ANALYSIS TECHNIQUES

By

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The almond moth, Cadra cautella (Walker), synthesizes alanine, aspartic acid, glutamic acid, glycine, proline, and serine from U-^{14}C-glucose. These amino acids are considered nutritionally non-essential. Amino acids that contained no radioactivity and are considered nutritionally essential include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine. Cystine and cysteine contained an intermediate amount of activity and are still unclassified with respect to dietary need.

Radioactive essential and non-essential free amino acids were isolated from larval rearing media. The specific activities were substantially higher in amino acids extracted from medium that had larvae reared on it than medium that had no larvae reared on it. There appears to be an insect-microorganism relationship at work here. However, the exact nature of this relationship and to

what extent it contributes to the insects' nutrition are at present undetermined. Since there was no detectable incorporation of radioactive essential free amino acids from the medium into the larval proteins (with the possible exception of cysteine), the importance of this relationship seems questionable when the techniques I employed for this study are used.

The study to isolate the source of the insect associated microorganisms is at present inconclusive. However, from the data available presently, it appears that the microorganisms come from within the almond moth eggs.

Carcass analysis of fifth-instar almond moth larvae showed that proline, tyrosine, and glutamic acid made up almost 70 percent of the total free amino acids present in the larvae. At this stage in larval development prior to pupation, cuticular tanning and thickening are beginning and proline and tyrosine are major participants in these events.

The protein amino acids, which contain a high percentage of glutamic acid and aspartic acid, in combination with the free amino acids are the basis for a dietary amino acid mixture at the 2 percent and 3 percent levels. By substituting this mixture for casein as the protein source in the almond moth diet, the requirements for vitamins and minerals can be determined more effectively.

INTRODUCTION

The study of insect nutrition, in particular the formulation of synthetic diets utilizing specific amino acids, vitamins, and minerals, has in recent years greatly expanded. When the specific nutrient requirements of an insect are known, many other facts about it become much clearer (i.e., physiological and biochemical). Once nutrition has been eliminated as a variable, many other aspects of the insect can be studied such as genetics, control (i.e., toxicant evaluation), biochemical relationships to higher or related organisms, etc.

Indirect nutritional procedures have come into widespread use to study organisms that cannot be reared axenically or on defined media. They also support and enhance information gained through classic nutritional techniques in addition to adding information on the metabolic pathways of many nutrients.

The indirect radioactivity method was first demonstrated in the work of Black, Kleiber, and Smith (1952) on the cow with radioactive carbonate and fatty acids being incorporated into non-essential amino acids. Shortly thereafter, Steele (1952) found that when U-^{14}C-sucrose was ingested by the adult mouse, radioactivity appeared in the non-essential but not in the essential amino acids extracted from the carcass proteins. Kasting and McGinnis (1958) confirmed this relationship for the

blowfly, Phormia regina (Meig), using U-^{14}C-glucose. Their data were supported by the results from the classical deletion procedure which had been done earlier on this insect.

The similarity in amino acid composition of the whole animal carcass and the pattern of amino acid requirements as determined by nutrition studies has been noted by Wu and Hogg (1952) for protozoa and Williams et al. (1954) for larger animals. The pattern of amino acids from carcass analysis studies has been used in several instances to formulate chemically defined diets with maximum larval growth being achieved (Auclair and Cartier 1963, Rock and King 1967b, and Rock and King 1967c).

The almond moth, Cadra cautella (Walker) (Lepidoptera: Pyralidae) is a common pest of tobacco, cocoa beans, dried fruits, and nuts throughout much of the world (Bach 1930, Wadsworth 1933, Fraenkel and Blewett 1946a). Therefore, it was chosen as the subject of this study. The objective of the study was to determine the qualitative (i.e., indispensable) amino acid requirements of the almond moth. This was to be followed by determination of the amount of each protein amino acid present in the carcass, with the aim of determining the quantitative amino acid requirements (both dispensable and indispensable) of this insect.

Once the qualitative and quantitative requirements had been established, a synthetic amino acid mixture could be developed.

By substituting this mixture for casein as the protein source in the almond moth diet, the requirements for vitamins and minerals can be determined more effectively. This is because dietary compounds such as casein often contain contaminants or substances which confuse nutritional research (i.e., vitamins, minerals, etc.). Once a completely defined diet has been established, nutrition could be eliminated as a variable factor in future studies on physiology, biochemistry, ecology, and ultimate control.

These two nutritional techniques were applied to my study of the almond moth.

LITERATURE REVIEW

Radioisotopes and the Determination of Amino Acid Requirements

Since the classic work of Steele (1952), many investigators have used carbon-14 labeled substrates to determine the nutritionally essential amino acids of a variety of different insects.

This procedure requires a compound that is normally present in the diet and readily metabolized as the carbon-14 substrate. Following administration by incorporation in the diet, injection, or some other suitable means, the organism is allowed time to metabolize the substrates.

Metabolism of the substrate results in incorporation of label in the synthesized compounds (i.e., nutritionally non-essential) and no incorporation in the essential compounds that must be supplied in the diet. (See Figure 1.)

The indirect radioactivity method has been applied to a number of phytophagus or other insects which cannot be reared on chemically defined media (Kasting and McGinnis 1958, 1960, 1962, 1964, Kasting et al. 1962, Strong and Sakamoto 1963, Rodriguez and Hampton 1966, Rock and King 1968, Rock and Hodgson 1971). A summary of the amino acid requirements of the above insects determined by this method is shown in Table 1.

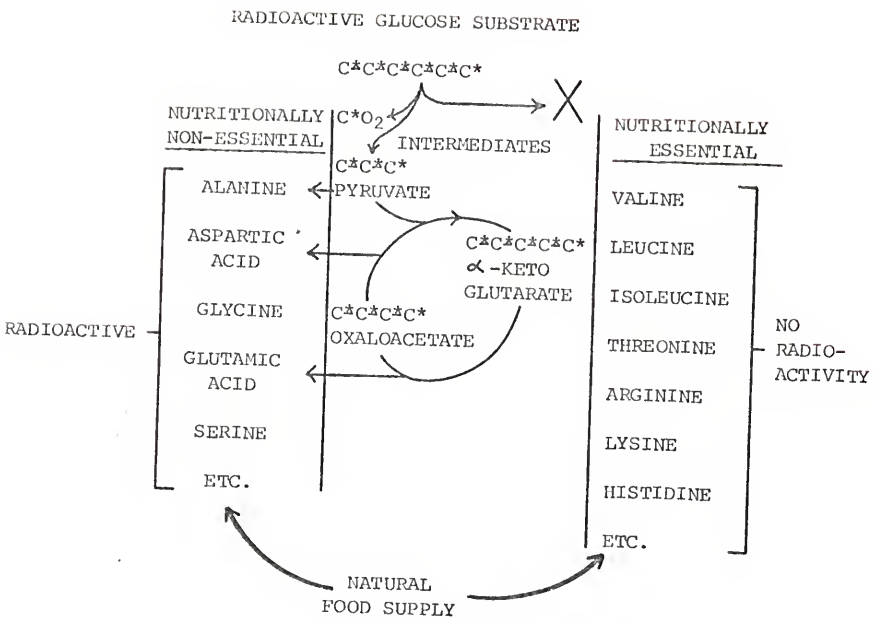

Figure 1. Principle of the radioactivity method for determining amino acid requirements.*

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* Kasting, R., and A. J. McGinnis. 1966. Radioisotopes and the determination of nutrient requirements. Ann. N.Y. Acad. Sci. 139: 99.

Table 1. Amino Acid Requirements of Some Insects Determined by the Radioactivity Method

Amino Acid	Blow Fly	Pale Western Cutworm	Wire-worm	Green Peach Aphid	Wheat Stem Sawfly	Two-Spotted Spider Mite	Red-Banded Leaf Roller	Boll Worm
Glutamic acid	-	-	+	-	-	-	-	-
Aspartic acid	-	-	-	-	-	-	-	-
Alanine	+?	-	-	-	-	-	-	-
Proline	+?	-	-		-	-	-	-
Serine	-	-	-	-	-	-	-	-
Glycine	-	-	-	-	-	-	-	-
Histidine	+	+	+	+	+	+	+	+
Threonine	+	+?	+	+	+	-	+	+
Leucine	+	+	+	+	+	+	+	+
Isoleucine	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	
Phenylalanine	+	+	+	+	+	+	+	
Lysine	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+
Methionine	+	+	+	+		+	+	+
Cystine	+	+		-		-		
Cysteine							-	-

+ = nutritionally essential
- = nutritionally non-essential
+?= some synthesis, possibly essential

There are several important factors to consider when using the indirect radioactivity method to determine nutrient requirements. The first of these is the method by which the radioactive substrate is administered. The labeled compound may be administered as a single dose or the organism may be continuously exposed to the radioactive substrate. Organisms are often continuously exposed to a radioactive substrate by having it incorporated in their diet (Strong and Sakamoto 1963, Rodriguez and Hampton 1966, Rock and King 1968, Rock and Hodgson 1971). Most studies involve the administration of only a single dose of substrate injection. Kasting and McGinnis (1964) have devised a method of vacuum infiltration for organisms that are not easily injected.

The radioactive substrate administered to the organism can affect both what compounds are labeled and the specific activity of these compounds. This was clearly shown in the study of Black et al. (1952) using bovine tissues. In this study, the labeling of amino acids varied depending on whether labeled acetate, propionate, butyrate, or bicarbonate was administered. Table 2 presents a summary of some of the substrates used, methods of administration, and metabolism periods for the determination of amino acid requirements using the indirect radioactivity method.

Table 2. Experimental Parameters Used to Determine Amino Acid Requirements by the Indirect Radioactivity Method

Organism	Radioactive Substrate	Method of Administration	Metabolism Period (Hours)	Source of Isolated Amino Acid	Reference
Cow	Acetate-I-^{14}C Acetate-II-^{14}C	Single injection	3, 10, 22, 34	Casein	Black et al. 1957
Frog & Tadpole	Glucose-U-^{14}C	Single injection	4, 8, 12	Liver, tail, muscle, carcass	Nakagawa et al. 1964
Mouse	Sucrose-U-^{14}C	Single feeding	72	Whole mouse (minus intestines)	Steele 1952
Rat	Glucose-U-^{14}C	Single injection	2.5 min.-2 hr.	Brain and liver	Gaitonde et al. 1965
Blow fly	Glutamic acid U-^{14}C	Single injection	48, 63	Whole carcass	Kasting & McGinnis 1960
Wheat Stem Sawfly	Glucose-U-^{14}C	Vacuum infiltration	24	Whole carcass	Kasting & McGinnis 1964
Green Peach Aphid	Glucose-U-^{14}C	Continuous feeding 24 hr.	24	Whole carcass	Strong & Sakamoto 1963
Red-Banded Leaf Roller	Glucose-U-^{14}C	Continuous feeding 48 hr.	48	Whole carcass	Rock & King 1968
Boll Worm	Glucose-U-^{14}C	Continuous feeding 48-72 hr.	60-84	Whole carcass	Rock & Hodgson 1970

The length of the metabolism period can affect the order of specific radioactivities among the protein and free amino acids. The level of specific radioactivity in free amino acids is not necessarily related to that of the protein amino acids. Nakagawa et al. (1964) showed that with a metabolism period of 4 hr. in the frog, the free amino acids that were labeled were not necessarily labeled in the protein amino acids from the same tissues. With a longer metabolism period following single administration of a radioactive substrate, there is generally greater incorporation of radioactivity into the protein amino acids, while radioactivity in the free amino acids may reach a peak and start to decline. The metabolism period often appears to be chosen by trial and error. Schaefer (1964) and Kasting et al. (1962) used the rate of production of $^{14}CO_2$ and the total amount produced following administration of radioactive substrates as a guide to selecting the optimum metabolic period.

To obtain reliable results using the radioactivity method, another factor that must be considered is the purity of isolated compounds (i.e., amino acids) before radioactivity measurement. This can be accomplished by simple two-dimensional thin-layer chromatography (TLC) with some organisms. However, with free amino acids from insect preparations, an ion exchange column followed by band paper chromatography in at least three solvent systems may be required to remove interfering materials (Kasting and McGinnis, 1960).

The tissues from which amino acids are isolated also have a significant effect on the results obtained with the indirect radioactivity method. Specific radioactivities will depend on whether amino acids are isolated from the free or protein fractions (Nakagawa et al., 1964), or the whole carcass or specific organs (Nakagawa et al. 1964, Black et al. 1952, Gaitonde 1965). However, with most organisms, the different synthetic abilities of different organs are not a problem because the amino acids are isolated from the whole animal. A problem arises when amino acids with low or intermediate specific activities are isolated from whole insect larvae. The low level of radioactivity in certain amino acids may be due to dilution of these compounds synthesized in one organ by unlabeled amino acids from other tissues or organs. Studies such as those carried out by Lipke et al. (1965) on the biosynthetic capabilities of the different tissues and organs of the cockroach will aid in interpretation of results by demonstrating which organs and tissues are capable of synthesizing specific amino acids.

The specific radioactivities of isolated amino acids may also vary depending on the concentration in which they are present. However, this has not been found with a number of insects (Kasting et al. 1962, Kasting and McGinnis 1962, 1964).

In many instances, the classical deletion technique has shown tyrosine to be a non-essential amino acid while the indirect

radiometric technique has indicated that it is essential (i.e., lacks radioactivity). Fukuda (1956) and Kasting and McGinnis (1962) have shown that tyrosine is synthesized from the essential amino acid phenylalanine and thus, even though it lacks radioactivity, it can still be classed as non-essential in many insects.

Kasting and McGinnis (1966) have thoroughly reviewed the subject of radioisotopes and the determination of nutrient requirements.

Carcass Analysis for the Estimation of Quantitative Amino Acid Requirements

To determine quantitative amino acid requirements of an insect, feeding tests on graded levels of an amino acid mixture, such as one resembling a casein hydrolysate or some other protein, are evaluated on the basis of growth and development of the insect.

A problem with this approach is that the initial balance of amino acids, both dispensable and indispensable, is probably far from optimum. Often, little consideration is given to the dispensable amino acid balance. Breuer et al. (1964) showed the importance of dispensable amino acid balance on total amino acid balance in the rat.

Several workers have used new methods to gain a better starting point from which to develop an optimum amino acid mixture for various organisms.

Wu and Hogg (1952) using protozoa and Williams et al. (1954) using the rat, chick, and pig noted the similarity in amino acid composition of the whole animal carcass and the pattern of amino acid requirements determined by nutritional studies. Auclair and Cartier (1963) successfully reared the pea aphid Acyrthosiphon pisum (Harris) on an amino acid diet based on the average concentration of these compounds in the blood and excreted honeydew. Rock and King (1967c) estimated the amino acid requirements for growth in the codling moth, Carpocapsa pomonella (Linneus) by carcass analysis. Rock and King (1967b) found that the quantitative pattern of amino acids in 1-day-old pupae of Argyrotaenia velutinana (Walker) supported maximum larval growth when this insect was reared axenically on a chemically defined diet. Rock and King (1966) studied the amino acid composition in hydrolysates of the red-banded leaf roller during development. Their work indicated a shift in amino acid requirements during growth and development. This shift in requirements is probably mediated by the requirements of the particular tissue that is being formed in the rapidly developing and growing larvae.

General Insect Nutrition

Several excellent reviews covering various phases of insect nutrition are currently available in the literature. Reviews concerning the general subject of insect nutrition include House (1961, 1962), Lipke and Fraenkel (1956), and Fraenkel (1959).

Friend (1962) discusses the nutritional requirements of phytophagus insects while House (1959) deals with the parasitoid Pseudosarco phaga affinis (Fall) and other insects. The current status of and future possibilities for research on the axenic culture of arthropods are discussed by Rodriguez (1966). Richards and Brooks (1958) and Henry (1962) reviewed the significance of internal symbiosis and microorganisms in insect nutrition. Fraenkel and Blewett (1946b, 1946c) and Waites and Gothilf (1969) have studied the dietary requirements of the almond moth and several other closely related lepidopterous insects.

Distribution and Metabolism of Amino Acids and Proteins

Numerous articles and reviews on the distribution and metabolisms of proteins and amino acids in insect tissues and fluids are available in the literature.

Florkin (1958) reviewed the subject of free amino acids in insect hemolymph and Chen (1962) the broader subject of free amino acids in insects. The free amino acids in Prodenia eridania, Culex pipiens, Glossina palpalis, and Drosophile melanogaster have been studied by Levenbook (1962), Chen (1963), Balogun (1969) and Mitchell and Simmons (1961), respectively.

In the area of amino acid metabolism, Brunet (1965) reviews the subject of aromatic compounds while Bheemeswar (1958)

discusses general amino acid metabolism covering the subject of major biochemical reactions (i.e., deamination, transammation, decarboxylation, and peptide and protein synthesis). Chen (1966) has written a very extensive review covering the subject of amino acid and protein metabolism in insect development. He follows the changing patterns of amino acid composition throughout the life stages of various insects. Henry and Block (1961) report on the metabolism of sulfur-containing amino acids in the German cockroach, Blattella germanica (L.). Bursell (1963) follows the changing pattern of free amino acids in the thorax of the male tsetse flies during the hunger cycle and flight activity.

Methodology

Thin-Layer Chromatography

Several two-dimensional solvent systems were investigated for the separation of complex amino acid mixtures before the one giving the best resolution was found.

Heathcote and Jones (1965) devised a pair of solvent systems for ascending two-dimensional chromatography on cellulose thin-layers. This system produced unambiguous separation of 23 naturally occuring amino acids, including leucine and isoleucine, in 6 hr. This method required no tank saturation and using a ninhydrin staining solution could detect less than 1 µg of amino acid.

The solvent systems were 2-propanol-formic acid-water (40:2:10 by volume) for development in the first dimension and tertiary butyl alcohol-methyl ethyl ketone-NH_4OH-distilled water (50:30:10:10 by volume) for development in the second dimension.

Jones and Heathcote (1966) used the same solvent system to separate the amino acids in protein hydrolysates. However, this time ninhydrin-collidine reagent was used to visualize the amino acids. This system made for easier identification of certain amino acids due to their characteristic color on staining.

Haworth and Heathcote (1969) modified their previous method and accomplished the separation of up to 63 compounds. This new solvent system consisted of 2-propanol-methyl ethyl ketone-1N HCl (60:15:25 v/v) for development in the first dimension and tertiary butyl alcohol-methyl ethyl ketone-acetone-methanol-NH_4OH-distilled water (40:20:20:1:14:5 v/v) for development in the second dimension. With this new solvent system, a large spread was produced between the amino acid spots. In addition, highly reproducible results were obtained making it possible to use the R_f's of the various spots to identify the amino acids present in a protein hydrolysate. Using a ninhydrin-cadmium acetate dye system 5×10^{-4} moles of amino acid can be detected following two-dimensional TLC. Heathcote and Haworth (1969a) again modified their solvent system to the following composition: tertiary pentyl alcohol-methyl ethyl ketone-acetone-methanol-

NH_4OH-distilled water (50:20:10:5:15:5 v/v). Heathcote et al. (1970) used selective staining to identify complex mixtures of amino acids and nitrogen containing metabolites separated by TLC.

De Zeeuw (1968a) compared the use of saturated and unsaturated TLC chambers. He obtained better separation using an unsaturated chamber in addition to obtaining good reproducibility of R_f's and good spot shape. Other factors that affect separation and spot shape and should be kept constant are temperature, relative humidity, solvents, adsorbent, and geometry of the chamber. De Zeeuw (1968b) also studied the influence of humidity variations on the TLC of hypnotics. He showed that R_f's changed considerably with variations in relative humidity of the TLC room. R_f's increased with increasing humidity and then fell sharply at higher humidities.

Heathcote and Washington (1967) and Heathcote and Haworth (1969b) discussed the quantitation of small amounts of amino acids separated by thin-layer or paper chromatography using colorimetric or densitometric techniques respectively.

Stahl (1968) stressed the need for standardization of terms in the literature so techniques could be repeated in other laboratories and results could be compared between laboratories.

Several excellent texts on TLC include Smith (1960), Stahl (1969), and Pataki (1966).

Gas Chromatography

A fairly recent advance in the quantitative analysis of amino acids was the adaptation of gas chromatography to these compounds. It is a very sensitive technique that requires only small amounts of the compound of interest to be injected into the instrument.

Gehrke et al. (1969) did an extensive study of the trimethylsilyl (TMS) derivatives of protein amino acids examining such factors as chromatographic separation, precision and accuracy of the method, silylation as a function of reaction temperature and time, molar excess of reactants, stability of the TMS derivatives, quantitative analysis of a synthetic amino acid mixture, and application to biological samples. Gehrke and Leimer (1970b) studied the effect of solvents on derivatization of amino acids using bis (trimethylsilyl) trifluoroacetamide (i.e., BSTFA). They found using polar solvents for the derivatization reaction produced two chromatographic peaks for glycine and one for arginine. In non-polar solvents, only the first chromatographic peak for glycine and no peaks for arginine were obtained. Gehrke and Leimer (1971) improved upon the previous work on trimethylsilylation of the 20 protein amino acids. Their major aim, which they accomplished, was to achieve a single derivatization, single injection method for the analysis of the 20 protein amino acids as the TMS derivatives in complex

biological substances. Amino acids were reproducibly converted in a single step, closed tube reaction to the TMS derivatives in 2.5 hr. at 150°C. Excellent separation of the 20 TMS protein amino acid derivatives was achieved on a single 6 m X 2 mm I.D. column packed with 10 percent OV-11 on 100/200 mesh Supelcoport in 60-80 min. Data from amino acid analysis of ribonuclease, B-casein, K-casein, soybean meal, and blood are in good agreement with values obtained by classical ion-exchange methods, and establishes the use of the TMS-/gas-liquid chromatographic (GLC) method for quantitative analysis of amino acids in biological materials.

Several other derivatives are available for amino acid analysis by GLC. Vance and Feingold (1970) and Pisano and Bronzert (1972) studied the methylthiohydantoin derivatives of amino acids and Fu and Mak (1971) the N-acyl amino acid alkyl esters.

Along with the TMS amino acids, the other major derivatives for GLC are the N-trifluoroacetyl (N-TFA) n-butyl ester and, in some cases, the methyl ester (Islam and Darbre 1969, Roach and Gehrke 1969a, 1969b, Casagrande 1970, Pellizzari et al. 1971, Gehrke et al. 1971).

Zumwalt et al. (1970) used the N-TFA n-butyl esters for quantitative analysis of amino acids in complex biological substances such as urine and blood plasma. He used ion-exchange

resins for sample clean-up and obtained quantitative recovery of amino acids from the exchange columns.

Gehrke and Leimer (1970a) studied the effect of salts on the derivatization and chromatography of N-TFA n-butyl esters of amino acids. They found that inorganic salt at a ratio of 1:1, salt to total amino acids, was not a serious problem for qualitative work. However, for quantitative work, the following salts should be removed by ion-exchange chromatography: oxalate, manganese (II), cobalt (II), nickel, zinc, tin (II), lead (II), chromium (III), and iron (III).

Gehrke et al. (1971) used the N-TFA n-butyl ester derivatives to search for amino acids in hydrolysates of lunar fines from Apollo 11 and 12 missions.

Zumwalt et al. (1971a, 1971b) refined the N-TFA n-butyl ester systems to the point where nanogram and picogram amounts of amino acids could be analyzed.

Gehrke et al. (1971) have an excellent review article on the TMS and N-TFA n-butyl ester systems for the analysis of the 20 protein amino acids in biological samples.

Burchfield and Storrs (1962) have a general text on the biochemical applications of gas chromatography.

Radioisotope Techniques

The use of radioisotopes in metabolic studies has greatly increased the number of scientific questions that can be answered and the limits of detection that can be reached.

Snyder (1965) reports on quantitative radioassay methods for TLC. He compares zonal versus autoradiographic scans and prefers zonal scans due to their greater resolving power and speed. Snyder (1966) also compares zonal versus strip scans of thin layer chromatograms and again prefers the zonal scans due to their greater sensitivity and resolving power. This is especially true with weak beta emitters such as ^{3}H-labeled compounds in biological specimens. Three excellent reviews covering the subjects of TLC radioassay, instrumentation and procedures for ^{14}C and ^{3}H radioassay by TLC and liquid scintillation radioassay of thin layer chromatograms are presented by Snyder (1968, 1969a, 1969b).

Bell and Hayes (1958) review many aspects of liquid scintillation counting.

Protein and Amino Acid Extraction and Treatment

Many texts are available covering the vast subject of protein and amino acid extraction, hydrolysis, clean-up, etc. Some of the more complete works include Block and Weiss (1956), Alexander and Block (1960a, 1960b), and Blackburn (1968).

Roach and Gehrke (1970) developed a new rapid acid hydrolysis technique for proteins. Using aqueous 6N HCl at a ratio of 1 mg. of protein to 1 ml. of acid, they heated the mixture in a sealed test tube containing a N_2 atmosphere for 4 hr. at 145°C. $\pm 2^{\circ}$. Essentially, equivalent hydrolysis and yield were obtained when this method was compared with the standard 110°C. $\pm 1^{\circ}$ for 26 hr. using ribonuclease as a protein source.

MATERIALS AND METHODS

Rearing

A stock culture of the almond moth was maintained on a diet consisting of the following ingredients: 4 parts cornmeal, 4 parts whole wheat flour, 2 parts finely ground dog food, 1 part brewer's yeast, 1 part oatmeal, 1/2 part wheat germ, 1 part honey, and 1 part glycerine. These ingredients were mixed thoroughly and placed in 1/2 gal. wide-mouth mason jars. The medium was then innoculated with several hundred eggs and the jars covered with 12 cm. filter paper discs and sealed with metal jar rings. Following emergence of adult moths, the filter paper discs were replaced with screen wire discs. The jars were then inverted in a rack and eggs were collected in petri dishes as they dropped through the screen. These eggs were used to innoculate the next generation of the culture.

Radiochromatography and Autoradiography of U-^{14}C-Glucose

Uniformly labeled ^{14}C-glucose was used throughout this study. The purity of the ^{14}C-glucose was checked by TLC and autoradiography of the chromatograms.

Twenty micrograms of unlabeled carrier glucose in a volume of 5 µl. was applied to an Eastman Chromagram Sheet of Silica Gel G. The spot was then dried in a cool air current. An aliquot of radio labeled glucose containing 0.01 µCi. of activity was then applied on top of the unlabeled carrier spot. In a second lane next to the ^{14}C-glucose, 10 µl. of an unlabeled 1/2 percent aqueous glucose solution was spotted.

The solvent system used for development was n-butanol-isopropanol-water (5:3:1). The chromatogram was allowed to develop until the solvent front reached 1/2 in. from the top edge of the plate. Following development, the chromatogram was thoroughly dried to remove all traces of solvent. The plate was then sprayed with a solution consisting of 0.9 g. oxalic acid and 1.8 ml. aniline in 200 ml. of H_2O and heated to 105°C. for 15 min. to visualize the glucose spot. The dried chromatogram was then wrapped in a single thickness of plastic film in order to protect the x-ray film from any substances on the thin-layer plate which might cause fogging. Then, in a dark room with a safety light on, a sheet of unexposed x-ray film was placed in direct contact on top of the plastic wrapped chromatogram. A small notch was cut in the film and chromatogram. The separated film and plate could then be realigned after development by aligning the notches. The film and chromatogram were then placed

between two pieces of wood cut approximately the same size as the x-ray film. The two pieces of wood were held together securely by placing several elastic bands around them. This procedure kept the film and chromatogram aligned correctly. The wood blocks were then wrapped in several layers of aluminum foil and placed in a drawer for 20 hr. Following this, the x-ray film was developed, washed, and air dried.

R_f's for the radioactive and non-radioactive glucose spots were then determined and compared and the x-ray film examined for the ^{14}C-glucose spot and any impurities or streaking.

Radioactive Medium Preparation

Radioactive medium was prepared by pipetting 160 µCi. of ^{14}C-glucose* onto 30 g. of standard rearing medium. The medium was thoroughly mixed and placed in an 8 oz. baby food jar. It was then innoculated with approximately 200 eggs and the jar sealed with a filter paper disc and metal jar ring. Following development, mature larvae were removed from the radioactive medium for extraction of protein.

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* Supplied by Amersham/Searle. Specific Activity - 309 mCi./mM.

Protein Extraction

A weighed amount of mature larvae that had been reared on medium containing ^{14}C-glucose was placed in a tissue grinder and homogenized with 20 ml. of cold 10 percent trichloroacetic acid (TCA). The grinder was rinsed with 10 ml. of cold 5 percent TCA and the liquid combined with the larval homogenate. The homogenate was then centrifuged for 5 min. and the supernatant decanted off for further analysis. The pellet containing the protein fraction plus other components (i.e., lipids, nucleic acids, etc.) was dried under a stream of N_2.

Twenty milliliters of acetone, made alkaline with NH_4OH, was added to the centrifuge tube. The mixture was then placed in a 70°C. water bath and stirred gently for 5 min. Following this, it was again centrifuged for 5 min. and the supernatant poured off and saved. The pellet was then dried under a stream of N_2. The acetone extraction, centrifugation, and drying steps were then repeated. The pellet was subjected to this treatment again, first using 95 percent ethanol and then using ether. It was repeated twice with each solvent and all supernatant fractions were saved for weighing and scintillation counting.

The pellet plus 10 ml. of 5 percent TCA were then placed in a 90°C. water bath for 15 min. with continuous stirring. The tube and its contents were then cooled under running water, centrifuged, the supernatant poured off and the pellet dried

under N_2. The protein pellet was then washed with 3-5 ml. portions of 5 percent TCA centrifuged, dried, and weighed.

The flow diagram in Figure 2 will help illustrate what components were extracted with the different solvents.

As a control, protein was extracted from the radioactive media. Two types of samples were analyzed; medium that had larvae reared on it and medium that had no larvae reared on it. This protein was extracted and analyzed in the same way as the insect protein.

Free amino acids from the media, which were contained in the first TCA supernatant fraction, were also analyzed. Before TLC could be used to separate the amino acids in this fraction, it first had to be cleaned up using the column method for carcass analysis of free amino acids.

Protein and free amino acids were also extracted from mature larvae that had been reared on non-radioactive medium. These were used in the carcass analysis study.

Protein Hydrolysis

Proteins extracted from the larvae and media were subjected to acid and base hydrolysis so analysis of the amino acids could be accomplished by TLC and GLC.

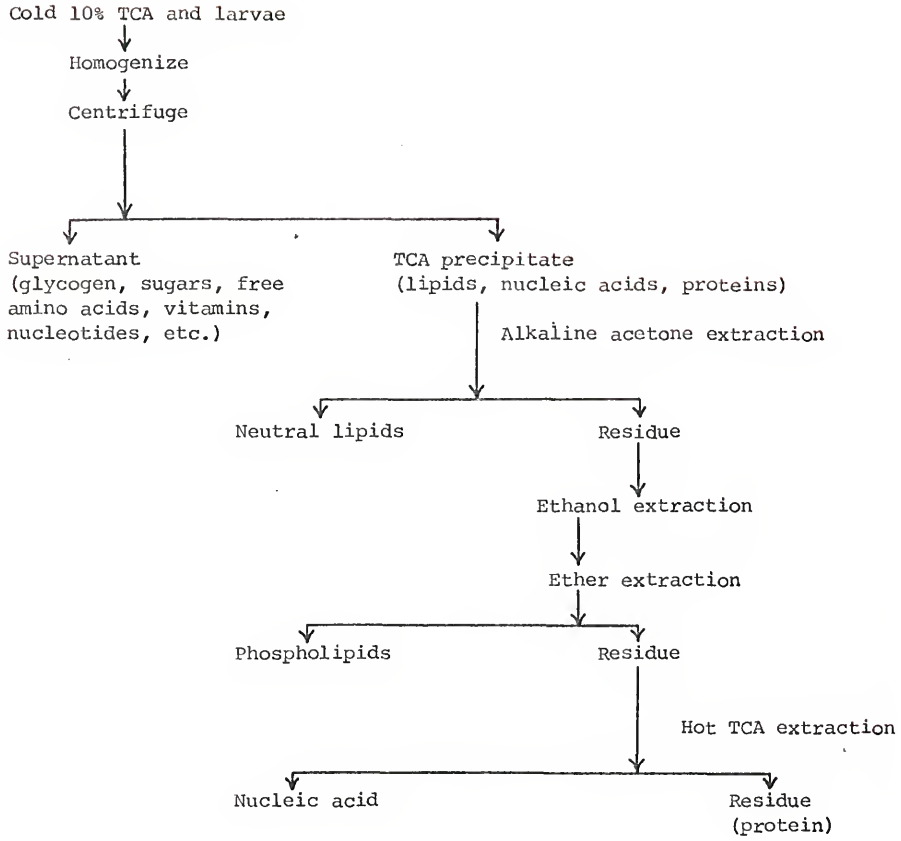

Figure 2. Flow diagram for the extraction and clean-up of proteins used in this study.

Acid Hydrolysis:

Ten milligrams of protein were placed in a 125 mm. screw top test tube with a Teflon-lined cap. The tube was flushed with a stream of filtered N_2 and 10 ml. of 6N HCl were added. The tube was again flushed with N_2, sealed, and heated for 4 hr. at 145°C.

The protein hydrolysate was then evaporated to dryness under vacuum in a 60°C. water bath. The residue was taken up in 2 ml. of 10 percent aqueous 2-propanol (v/v) and again evaporated to dryness. This step was repeated once again after which the residue was dissolved in 1/4 ml. of 10 percent aqueous 2-propanol for TLC. For GLC analysis, the amino acid residues were dissolved in 1 ml. of 0.05 N aqueous HCl.

Base Hydrolysis:

Base hydrolysis was used for the study of amino acids that were partially or completely destroyed by acid hydrolysis, such as tryptophan. Ten milligrams of protein, 65 mg. of $Ba(OH)_2 \cdot 8H_2O$ and 1 ml. of H_2O were placed in a screw top test tube. The top of this tube had a small hole drilled in it and a silicone rubber septum from a gas chromatograph injection port was placed in the top. The top was screwed on and a hypodermic needle that had been attached by means of rubber tubing to a vacuum line was inserted into the tube through the hole in the top. The tube was then evacuated with the rubber septum keeping it air tight.

The tube was heated for 24 hr. at 125°-130°C. after which it was cooled. The protein hydrolysate was then adjusted to pH 6 with 2N H_2SO_4 and then heated to boiling. The tube and its contents were then centrifuged to separate the $BaSO_4$. The $BaSO_4$ pellet was washed with a little water and the combined supernatant and washing were evaporated to dryness. The residue was then dissolved in 1/4 ml. of 10 percent aqueous 2-propanol for separation of the amino acids by TLC.

Thin-Layer Chromatography of Amino Acids

Amino acids from the protein hydrolysates were separated and identified by TLC. Five microliters of hydrolysate were spotted on a 20 X 20 cm. Eastman Chromagram Sheet of cellulose without fluorescent indicator. The starting point was 1/2 in. from the edges of the plate at the bottom left hand corner. The spot was positioned by marking the edges of the plate with a soft lead pencil. The solvent front was also marked in this manner, care being taken so as not to disturb the thin-layer and cause distortion of the spots during chromatography. After application to the thin-layer plate, the spot was dried in a stream of warm air.

Separation of 20 amino acids required the use of two-dimensional chromatography. The solvent systems used were 2-propanol-methyl ethyl ketone-1N HCl (60:15:25 v/v) for

development in the first dimension and 2-methyl-2-butanol-methyl ethyl ketone-acetone-methanol-water-concentrated NH_4OH (50:20:10:5:15:5 v/v) for development in the second dimension. Development in each phase was allowed to continue until the solvent front reached 1/2 in. from the top edge of the plate. Between development in the first and second dimensions, the plate was dried for 2 hr. in a fume hood. Following development in the second dimension, the plate was allowed to dry overnight.

Visualization of the amino acids was accomplished by spraying the dried plates with 1/2 percent ninhydrin in acetone and then heating for 20 min. at 60°C.

A standard plate (i.e., spot map) was prepared to facilitate identification of the amino acids. Standard solutions (0.025 M) of the 22 amino acids of interest were made up in 10 percent aqueous 2-propanol. These standards were chromatographed and their positions on the plate noted along with their R_f's in both dimensions.

Radioactivity Measurements

The supernatant fractions from the larval and media protein extractions were placed in numbered scintillation vials that had been previously weighed. The liquid was then evaporated to dryness under a N_2 stream and heat lamp and the vials again weighed. After the weight of each supernatant fraction was

known, 1 or 2 ml. of Soluene,* a sample solubilizer, was added to each vial. The samples were set aside for 48 hr. to allow the solubilizer to work. Fifteen milliliters of scintillation fluid consisting of 5 g. 2,5-diphenyloxazole (PPO), 0.250 g. 1,4 bis-2-(4-methyl-5 phenylox-axolyl)-benzene (dimethyl POPOP), and 1 L. of toluene were added to each vial. The vials were then placed in a Packard Tri-Carb Liquid Scintillation Spectrometer and allowed to temperature equilibrate (3-4^{o}C.) for 1 hr. These samples were counted for only 10 min. due to the high activity present. The counts per minute (cpm) were corrected for any quenching with automatic external standardization (AES).

Following separation and visualization, the individual amino acids were scraped off the thin-layer plates into scintillation vials. The spots from 10 plates were pooled for each amino acid. For scintillation counting, a cocktail similar to the previous one was used except that Cab-O-Sil** (4 percent w/w) was added. Cab-O-Sil is a gelling agent which aided in suspension of the amino acids on the thin-layer adsorbent. The vials were then placed in the scintillation counter and allowed to temperature equilibrate for 1 hr. They were counted for 100 min.

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* Packard Instrument Company, Inc.

** Rohm and Haas.

AES was used to correct the counts for any quenching in the samples. Several vials containing different amounts of thin-layer adsorbent were also counted to determine if the adsorbent caused any increase in activity due to fluorescence.

The quenching properties of 20 amino acids and the supernatant fractions from the larval protein extraction were studied using internal standardization. This study was undertaken to test the validity of the external standards method.

Twenty microliters of a 1 mg./ml. standard of each of 20 amino acids were pipetted onto a cellulose thin-layer plate that had been divided into 20 sections. Each section of the plate contained a single amino acid standard. Twenty scintillation vials each containing 15 ml. of the same scintillation cocktail used in the counting of amino acids from the protein hydrolysate, 10 µl. of ^{14}C-glucose, and 2-3 drops of Bio-solv^{TM}* solubilizer were counted in a Beckman LS-200 Liquid Scintillation Spectrometer prior to the addition of a single amino acid; each vial was recounted to see how much quenching resulted.

This same study was repeated again with the exceptions that this time only 5 µl. of ^{14}C-glucose were used and the amino acids were visualized by the method noted previously before they were added to the scintillation vials.

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* Beckman Instrument Company.

The quenching properties of supernatants from the protein extractions were studied in a similar manner. The supernatant fractions, from the extraction of protein from larvae reared on non-radioactive medium, were dried, weighed, and solubilized as previously discussed. Each one was then added to a separate vial which contained 15 ml. of the same scintillation fluid used previously for counting supernatant fractions. These vials also contained 10 µl. of ^{14}C-glucose and 1-2 drops of Bio-solv^{TM} solubilizer. They had been counted before the addition of the supernatant fractions and were now counted a second time to determine the extent of quenching caused by the various fractions.

Carcass Analysis

Fifth-instar larvae were analyzed for the total amount of each amino acid they contained. The protein and free amino acids were analyzed separately and then combined later to arrive at a total for each individual amino acid. The carcass analysis was replicated twice using two separate groups of larvae.

Protein extraction and subsequent hydrolysis for the liberation of amino acids were accomplished using the same method as described previously for the thin-layer work. The free amino acids were obtained from the supernatant of the larval-TCA homogenate following centrifugation. Before the free amino acids could be derivatized for GLC, they had to be separated from any

interfering biological substances with an ion-exchange column.

The resin, Amerlite CG-120 (100/200 mesh), was prepared as follows: resin was placed in a 500 ml. beaker and covered with 3N NH_4OH. It was then placed on a magnetic stirrer and swirled for 60 min. The resin was allowed to settle and the NH_4OH decanted off. This process was repeated twice more and the resin was then washed with double distilled water until it was approximately neutral. The resin was then regenerated by swirling for 30 min. three times with 3N HCl. It was then washed with double distilled water until it was approximately neutral. The columns, which consisted of 125 mm. test tubes with a 2 mm. hole in the bottom, were then filled to the 3/4 mark with wet resin. The level of liquid was never allowed to fall below the surface of the resin. The liquid in the column was then allowed to fall to approximately 3 mm. above the resin surface and the sample was added with a pasteur pipette. The entire 30 ml. of TCA supernatant fraction were passed through the column. Following this, 5-10 ml. portions of distilled water were used to wash the resin. The washes were discarded. The amino acids were then eluted from the column using five separate 2 ml. portions of 3N NH_4OH. This was followed by five, 5 ml. portions of distilled water. The flow rate through the column was approximately 1-2 ml./min.

The effluent from the column was collected in a 125 ml. round bottom flask. It was evaporated to dryness on a rotary evaporator with the flask immersed in a 60°C. constant temperature water bath. The residue was dissolved in 1 ml. of aqueous 0.05N HCl for derivatization. Free amino acids extracted from the rearing media were dissolved in 1/4 ml. of 10 percent aqueous 2-propanol for TLC.

Gas Chromatography of Amino Acids for Carcass Analysis

Column Packing and Preparation:

Twenty grams of Supelcoport* 100/200 mesh were placed in a round bottom flask and just covered with methylene chloride. The methylene chloride had been dried by running it through a silicic acid column and was then distilled into an all glass bottle to protect it from atmospheric moisture.

OV-11 (2.22 g.) dissolved in a minimal amount of methylene chloride, was then added to the round bottom flask containing the Supelcoport. This gives a 10 percent loading of OV-11 on the solid phase. The flask was then placed on a rotary evaporator

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* Supelco Inc.

and the methylene chloride slowly evaporated at room temperature until the column packing was just damp. The flask was then immersed in a 60°C. water bath while under full vacuum on the rotary evaporator until no odor of methylene chloride remained.

A 12 ft. by 2 mm. I.D. glass column was then silylated to prepare it for the column packing. The column was first filled with a 10 percent v/v solution of dimethyldichlorosilane in toluene and allowed to stand for 15 min. with the solution in it. The column was then flushed and filled with absolute methanol. After 5 min., the methanol was removed and the column was washed twice with acetone. It was then placed in an oven to dry. The packing of 10 percent OV-11 on 100/200 mesh Supelcoport was then added to the column.

The column was then placed in the gas chromatograph oven and flushed with N_2 carrier gas for 30 min. Following this, it was no-flow conditioned at 325°-330°C. for 12-15 hr. The oven was then cooled to room temperature. A flow of 10-15 ml./min. of N_2 carrier gas was used for the rest of the conditioning. The oven was then temperature-programmed to 300°C. at a rate of 1°C./min. and allowed to remain undisturbed at this temperature for at least 24 hr.

Derivatization of Amino Acids:

An aqueous aliquot of protein hydrolysate or free amino acid extract, containing from 0.5-6 mg. of total amino acids, was

added to a 65 mm. screw top culture tube with a Teflon-lined cap. The amino acid solution was just evaporated to dryness in a 70°C. sand bath while passing a stream of regulated, filtered N_2 into the tube. Methylene chloride (0.5 ml.) was then added to the tube and evaporated just to dryness. This last step was repeated two more times. A known amount of internal standard, in this case decanoic acid in acetonitrile, was then added to the tube. The amount of internal standard should correspond to about the amount of each individual amino acid in the test tube and there should be 0.25 ml. of acetonitrile for each mg. of total amino acid. Therefore, in the standard tube which contained 0.1 mg. of each of 20 amino acids for a total of 2 mg. of amino acid, an internal standard of 0.2 mg./ml. acetonitrile would be used. By adding 0.5 ml. of this solution, the required 0.1 mg. of internal standard and 0.5 ml. of acetonitrile for the 2 mg. of total amino acid would be added.

In derivatizing the protein hydrolysates, an aliquot corresponding to 4 mg. of total amino acids was used. Since the amount of each amino acid in the hydrolysate varied, 0.1 mg. of internal standard was chosen as the arbitrary amount to use.

An additional problem with the free amino acid extracts was that the amount of total amino acids was not known. Therefore, these several equal aliquots were derivatized using different total amounts of acetonitrile.

Following addition of the internal standard, 0.25 ml. of bis (trimethylsilyl) trifluoroacetamide (BSFTA) was added for each 1 mg. of total amino acids in the tube. Different amounts of BSFTA were also tried in the free amino acid derivatizations. The tubes were then securely closed and placed in an ultrasonic bath for 1 min. to insure complete mixing.

The trimethylsilyl (TMS) derivatives of the amino acids were made by heating the tubes for 2.5 hr. at 150°C. in an oil bath. The tubes should be only 1/4 full and not immersed in the oil above the level of liquid. A reagent blank containing everything but amino acids was also run to check for extraneous peaks.

In addition to the protein hydrolysates and free amino acid extracts which were used for carcass analysis, several other samples were studied. Supernatant fractions from the extraction of protein from non-radioactive larvae were studied to see if there was any loss of amino acids during the extraction procedure. The first of each of the following fractions were studied: alkaline acetone, ethanol, ether, and hot TCA. These fractions were cleaned up using the same procedure as that used for the larval free amino acids.

A second larval protein extraction was then performed and the same fractions were analyzed. However, this time the fractions were evaporated to dryness and then hydrolyzed with 6N HCl. The hydrolysates were then cleaned up using the same column procedure

as that for the larval free amino acids. Both sets of fractions were then derivatized in the same manner as the protein hydrolysate samples.

Gas Chromatography of Trimethylsilyl Amino Acids:

The carcass analysis samples were chromatographed on a model 2100 Varian Aerograph using a flame ionization detector. Five microliters of sample were injected directly onto the column. The following parameters were used for the separation and detection of amino acids: injector temperature, 275°C.; detector temperature, 300°C.; N_2 carrier gas flow rate, 17 ml./min.; oven temperature, initial 100°C.; 3-min. hold after start of solvent peak, 4°C./min. increase to 300°C.; attenuator settings, 32 X 10^{-11}.

The samples prepared from the various protein extraction supernatants were analyzed on a Tracor MT 220 gas chromatograph using a flame ionization detector. The following chromatographic conditions were used with this machine: injector temperature, 270°C.; detector temperature, 225°C.; N_2 carrier gas flow rate, 17 ml./min.; oven temperature, initial 100°C.; 3-min. hold after start of solvent peak, 5°C./min. increased to 210°C.; attenuator settings 8 X 10.

Microbiological Study

When analysis of several free amino acid fractions from radioactive media, both with and without larvae on them, showed radioactive amino acids to be present, a study was undertaken to find the source of their synthesis.

The third replicate of the essential amino acid study was run under aseptic conditions. The larval rearing medium, containing ^{14}C-glucose, was heat sterilized at 15 lb. pressure for 15 min. The medium was sterilized in the rearing jar, which was covered with aluminum foil. Almond moth eggs were surface sterilized by placing them in a 3 percent zephiran chloride solution, in sterile distilled water, for 15 min. The eggs were rinsed well with sterile distilled water and put on sterile filter paper in a sterile petri dish. All work was carried out in a sterile hood. The eggs were then placed on the sterile, radioactive medium and the rearing jar was placed in a closed TLC tank to minimize air movement and maintain a sterile environment. The free amino acids in this rearing medium were analyzed after the larvae were taken off for protein extraction. The free amino acids from both sterile and non-sterile medium, containing ^{14}C-glucose but no larvae, were also extracted and analyzed as a control.

The sterility of the almond moth eggs and radioactive medium was checked by incubating them separately in nutrient broth at 37°C. for 4 days and then streaking on nutrient agar plates. The

plates were then incubated at 37°C. for 4 days and read as + (i.e., growth) or - (i.e., no growth). Other materials studied in this way or simply by streaking on nutrient agar plates were: non-sterile eggs, non-sterile medium, sterile larvae, and sterile medium that had sterile larvae reared on it. Three replicates were run on each material studied.

RESULTS AND DISCUSSION

Qualitative Amino Acid Requirements by The Indirect Radioactive Method and Thin-Layer Chromatography

Two-dimensional TLC permitted the identification of 19 amino acids (Figures 3 and 4) from acid and base hydrolysates of protein from fifth-instar almond moth larvae. The larvae had fed *ad libitum* on medium made radioactive with ^{14}C-glucose for approximately 3 weeks. Identification of amino acid spots was made with the aid of R_f and $R_{leucine}$ values (Table 3) calculated from a spot map (Figure 5) of 22 amino acid standards. The cpm/carbon atom (Table 4) of amino acids isolated from larval protein shows that alanine, aspartic acid, glutamic acid, glycine, proline, serine, and an unknown ninhydrin positive compound from the acid hydrolysis fraction were highly labeled. Because these amino acids were synthesized from glucose by the almond moth, they are considered nutritionally non-essential. The cpm/carbon atom of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, tyrosine, and two ninhydrin positive unknowns were not significantly above background. These amino acids were therefore not synthesized to any appreciable extent from glucose and must be considered nutritionally essential. Cystine and cysteine contained an intermediate amount of radioactivity. This

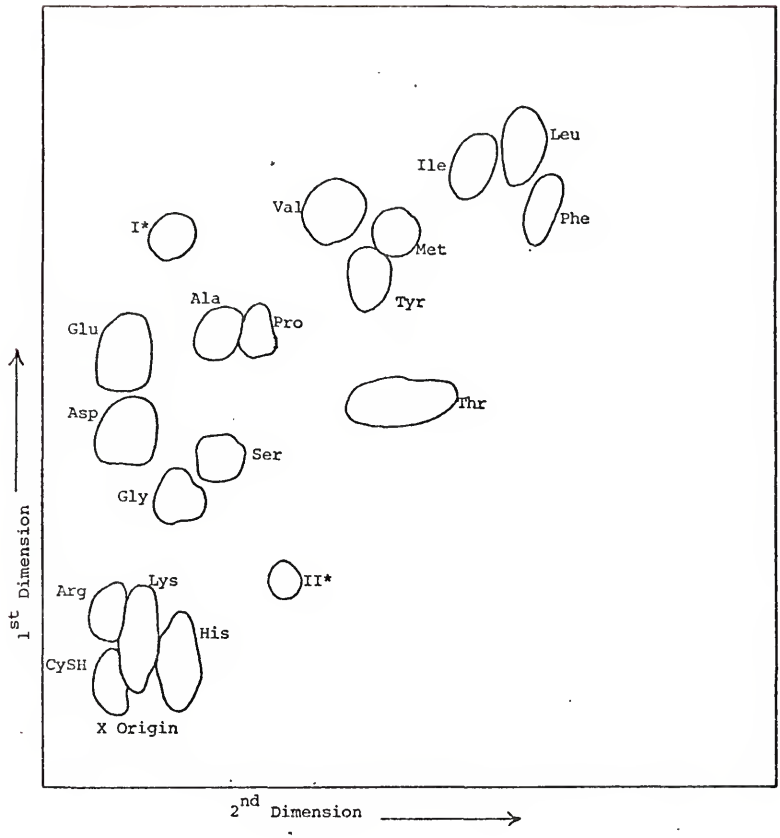

Figure 3. Separation of amino acids present in an acid hydrolysate of protein extracted from fifth-instar almond moth larvae reared on medium containing ^{14}C-glucose.

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* Unknown ninhydrin positive compounds.

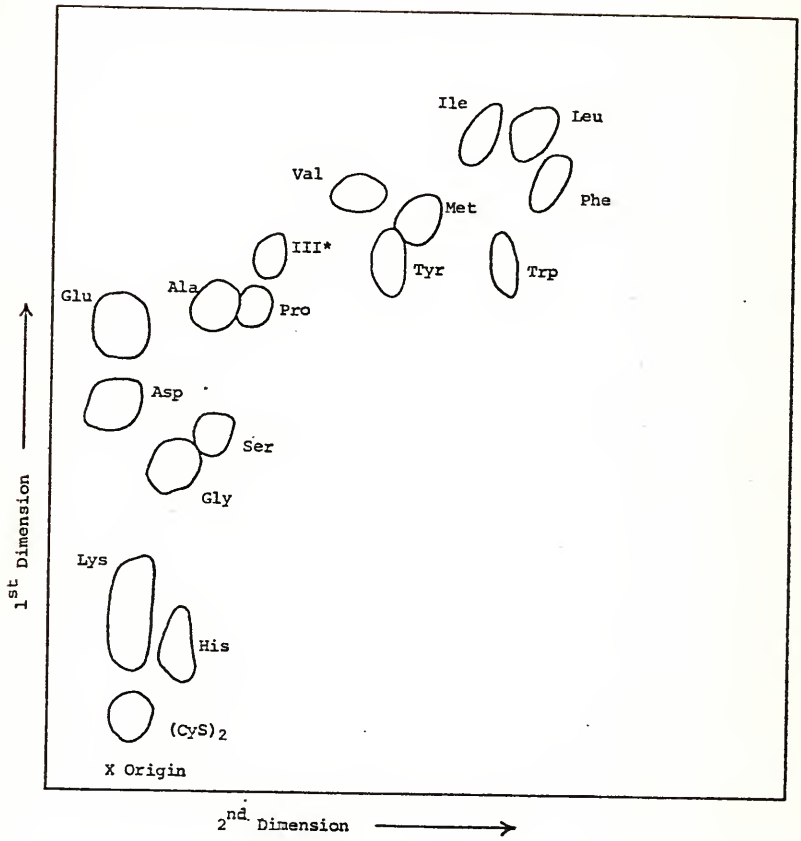

Figure 4. Separation of amino acids present in a base hydrolysate of protein extracted from fifth-instar almond moth larvae reared on medium containing ^{14}C-glucose.

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* Unknown ninhydrin positive compound.

Table 3. R_f and $R_{Leucine}$ Values of 22 Amino Acid Standards Separated by Two-Dimensional Thin-Layer Chromatography on Cellulose

Amino acid	First dimension*		Second dimension**	
	R_f X 100	$R_{leucine}$ X 100	R_f X 100	$R_{leucine}$ X 100
Alanine	55	63	12	25
Arginine	16	18	4	8
Asparagine	20	23	7	15
Aspartic acid	42	48	3	6
Cysteine	8	9	2	4
Cystine	4	5	2	4
Glutamic acid	52	60	3	6
Glutamine	26	30	8	17
Glycine	34	39	9	19
Histidine	9	10	13	27
Hydroxyproline	42	48	10	21
Isoleucine	86	99	44	92
Leucine	87	100	48	100
Lysine	15	17	8	17
Methionine	74	85	37	77
Phenylalanine	78	90	50	104
Proline	55	63	16	33
Serine	37	43	15	31
Threonine	46	53	40	83
Tryptophan	64	74	45	94
Tyrosine	69	79	31	65
Valine	76	87	29	60

* First dimension = 2-propanol-methyl ethyl ketone-1N HCl (60:15:25, v/v).

** Second dimension = 2-methyl-2-butanol-methyl ethyl ketone-acetone-methanol-water-ammonium hydroxide (50:20:10:5:15:5).

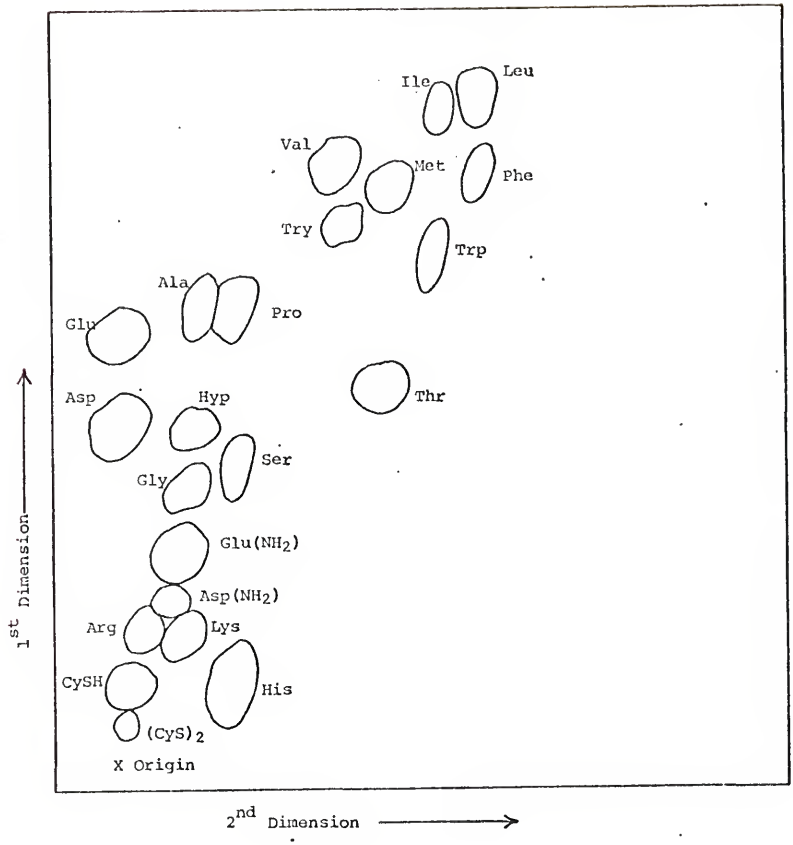

Figure 5. Spot map of 22 amino acid standards separated on cellulose thin-layer plates. Solvent system: first dimension: 2-propanol-methyl ethyl ketone - 1N HCl (60:15:25, v/v); second dimension: 2-methyl-2-butanol-methyl ethyl ketone-acetone-methanol-water-concentrated ammonium hydroxide (50:20:10:5:15:5, v/v).

Table 4. Cpm/Carbon Atom of Amino Acids from Acid and Base Hydrolysates of Protein Extracted from Almond Moth Larvae Reared on Medium Containing ^{14}C-Glucose

Amino acid	Carbon atoms/ amino acid	Mg. of amino acid/ 10 TLC plates	Cpm+/carbon atom		
			Rep. I	Rep. II	Rep. III
Alanine	3	.125	112.5	532.5	276.0
Arginine	6	.075	1.0	0.5	0.5
Aspartic acid	4	.211	62.0	270.7	86.0
Cysteine	3	-	11.0	16.0	13.5
Cystine	6	-	5.0	10.8	5.8
Glutamic acid	5	.271	74.0	328.5	116.5
Glycine	2	.070	81.0	347.0	182.0
Histidine	6	-	0.0	0.0	0.0
Isoleucine	6	.102	0.0	0.0	0.0
Leucine	6	.154	0.0	0.0	0.0
Lysine	6	.128	0.0	0.3	0.3
Methionine	5	.030	1.2	2.6	2.6
Phenylalanine	9	.081	0.1	0.2	0.0
Proline	5	.078	15.5	63.8	30.8
Serine	3	.083	55.0	256.5	25.5
Threonine	4	.059	1.3	0.0	0.0
Tryptophan	11	-	0.7	0.9	0.9
Tyrosine	9	.103	0.2	0.0	0.0
Valine	5	.109	0.4	0.0	0.0
Unknown I - acid hydrolysate*	4	-	33.7	41.0	41.0
Unknown II - acid hydrolysate*	4	-	0.8	0.0	0.0
Unknown III - base hydrolysate*	4	-	2.2	3.1	2.6

+ Cpm corrected for background and quenching.
* Calculated on the basis of four carbon atoms per molecule.

situation could indicate one of several possibilities. These two amino acids may be synthesized to only a limited extent and still need to be supplied in the diet. Another possibility is that dietary cysteine or methionine may spare the need for biosynthesis. There may actually be no radioactivity in these compounds and the activity observed may be coming from labeled contaminants which have been detected at the origin of the thin-layer plate by liquid scintillation counting. Only cysteine was found in the acid hydrolysis fraction. The cystine, if present, may have been substantially destroyed by the hydrolysis procedure and/or converted to cysteine (Blackburn 1968). Only cystine, or what appeared to be cystine, was found in the base hydrolysis fraction which is unusual since this procedure readily destroys both cysteine and cystine (Blackburn 1968). These two compounds are often found to be among the lowest in concentration of any amino acids present in the insect which complicates their detection and quantitation (Strong and Sakamoto 1963, Rodriguez and Hampton 1966, Rock and King 1968, and Rock and Hodgson 1971). Even though many insects do not require cysteine or cystine (Strong and Sakamoto 1963, Rodriguez and Hampton 1966, Rock and King 1968, and Rock and Hodgson 1971), I would supply these amino acids in the diet until further study could be done for the following reasons. The three carbon amino acids cysteine, serine, and alanine which are derived from pyruvate, should have a very high specific activity if they are indeed synthesized (Black et al. 1957, Rock and King 1968). Dilution

with unlabeled carbon, and thus low specific activity, would be expected at the level or stage of metabolism where proline is synthesized. The synthetic route to the carbon chain of proline is indirect and multiple intermediates exist where dilution of labeled carbon would be expected from unlabeled dietary components. Also, these amino acids being present in such low concentrations, as was noted previously, precludes extensive dilution of labeled cysteine and cystine with unlabeled cysteine and cystine.

Tyrosine, which contained no radioactivity, was not synthesized from the carbon chain of glucose. It must, therefore, be classified as nutritionally essential. Phenylalanine is known to be the principal precursor of tyrosine in the rat (Steele 1952). In insects, the synthesis of tyrosine from phenylalanine has been demonstrated in the silkworm larvae (Fukuda 1956), the pale western cutworm (Kasting and McGinnis 1962), and the prairie grain wireworm (Kasting et al. 1962). In the above cases when phenylalanine is supplied in sufficient amounts, tyrosine can be classified as nutritionally non-essential.

As a control, protein was extracted from two types of radioactive media; one medium had larvae reared on it and one medium had no larvae reared on it. Figures 6 and 7 are spot maps of an acid and base hydrolysate of proteins extracted from larval rearing media. Table 5 presents the data from radiometric analysis of these proteins. There was no synthesis of any radioactive protein amino acids in the medium by any organism which might have contributed to the activity in the proteins of the insect.

Several extractions of the larval rearing media for free amino acids were attempted. However, upon TLC, no satisfactory separation of amino acids was achieved. The amino acids tended to clump in three undistinguishable groups. These groups were pooled, counted by liquid scintillation, and found to contain activity. The ion exchange clean-up procedure which was used in the carcass analysis section of this paper was then tried prior to TLC of these free amino acids. This procedure resulted in a clean preparation, which upon TLC produced a high resolution, unambiguous separation of the amino acids present. Readable thin-layer plates were only obtained from the third replicate of this study which was carried out under aseptic conditions. The free amino acids from a sample of each of three radioactive media were studied. These were sterile medium that had larvae reared

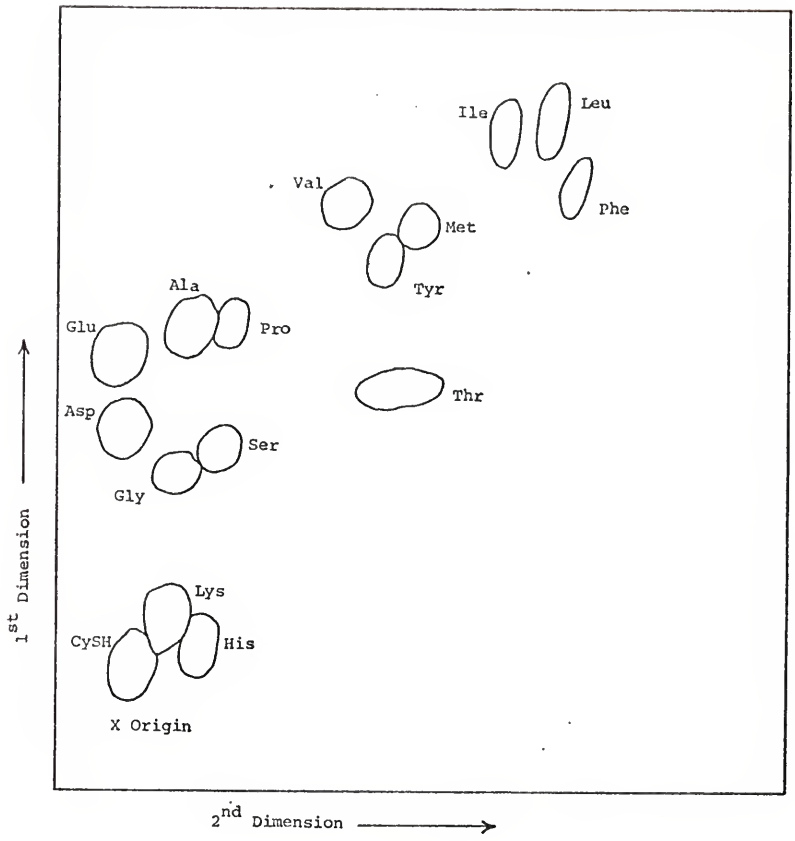

Figure 6. Separation of amino acids present in an acid hydrolysate of protein extracted from larval rearing medium which contained ^{14}C-glucose and on which larvae were maintained. The same pattern and amino acids were present in an acid hydrolysate of protein from radioactive medium on which no larvae were reared.

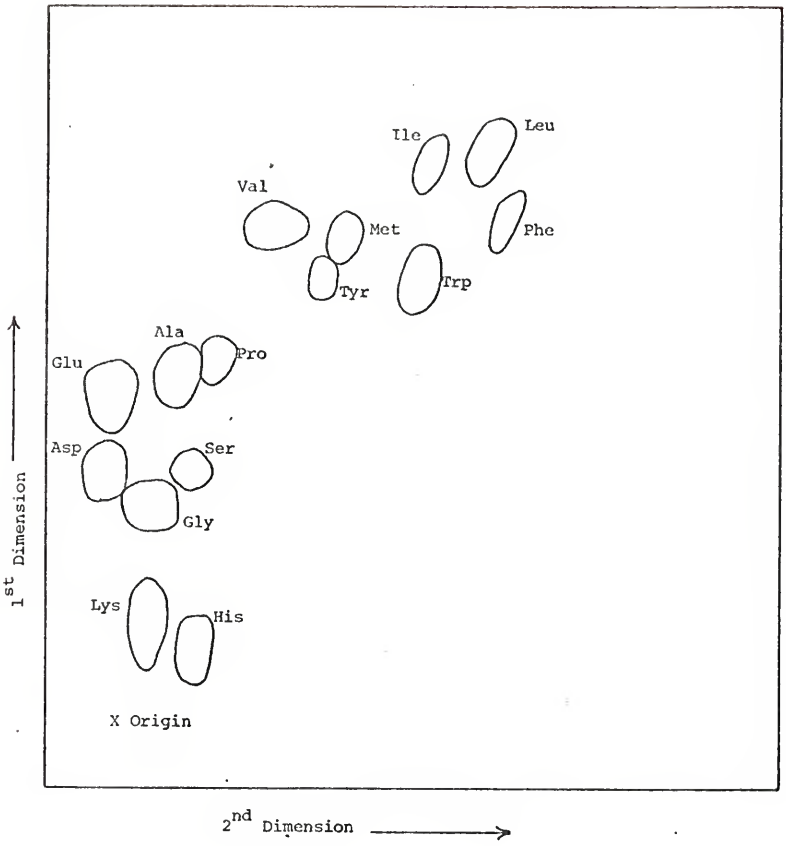

Figure 7. Separation of amino acids present in a base hydrolysate of protein extracted from larval rearing medium which contained ^{14}C-glucose and on which larvae were maintained. The same pattern and amino acids were present in a base hydrolysate of protein from radioactive medium on which no larvae were reared.

Table 5. Radioactivity in Amino Acids from Acid and Base Hydrolysates of Proteins Extracted from Larval Rearing Media Containing ^{14}C-Glucose

Amino acid	Medium containing larvae cpm/carbon atom*	Medium containing no larvae cpm/carbon atom*
Alanine	0	0.2
Aspartic acid	0	0
Cysteine	0	0
Glutamic acid	0	0
Glycine	0	0
Histidine	0	0
Isoleucine	0.1	0.1
Leucine	0.3	0.2
Lysine	0	0
Methionine	0	0
Phenylalanine	0.3	0.4
Proline	0	0
Serine	0	0
Threonine	0.3	0
Tryptophan	0	0.2
Tyrosine	0.3	0
Valine	0	0.3

* Corrected for background and quenching. Average of three replicates.

on it, non-sterile medium that had no larvae reared on it, and sterile medium that had no larvae reared on it. Figures 8, 9, and 10, respectively, show the amino acids present in these samples of media. Tables 6, 7, and 8 present the results of radiometric analysis of these amino acids. The amino acids extracted from sterile medium which had no larvae reared on it contained no radioactivity. This finding is self-explanatory and requires no further comment. The amino acids from non-sterile medium, which also had no larvae reared on it, contained substantial radioactivity. All 15 amino acids and the four ninhydrin positive unknown compounds contained activity to one degree or another.

Most microorganisms are known to be capable of synthesizing all the protein amino acids. Even though the larval rearing medium is quite dry and this should preclude the need for aseptic conditions (Fraenkel 1959), the synthesis of amino acids that is occurring is probably microbial in origin.

The free amino acid extract from the sterile medium that had larvae reared on it contained 22 free amino acids and five ninhydrin positive unknowns, all but one substantially labeled. The activity of the amino acids in this extract ranged from 3 to 28 times the activity in the corresponding amino acids from the non-sterile extract. The presence of almond moth larvae from surface sterilized eggs in the sterile rearing medium somehow

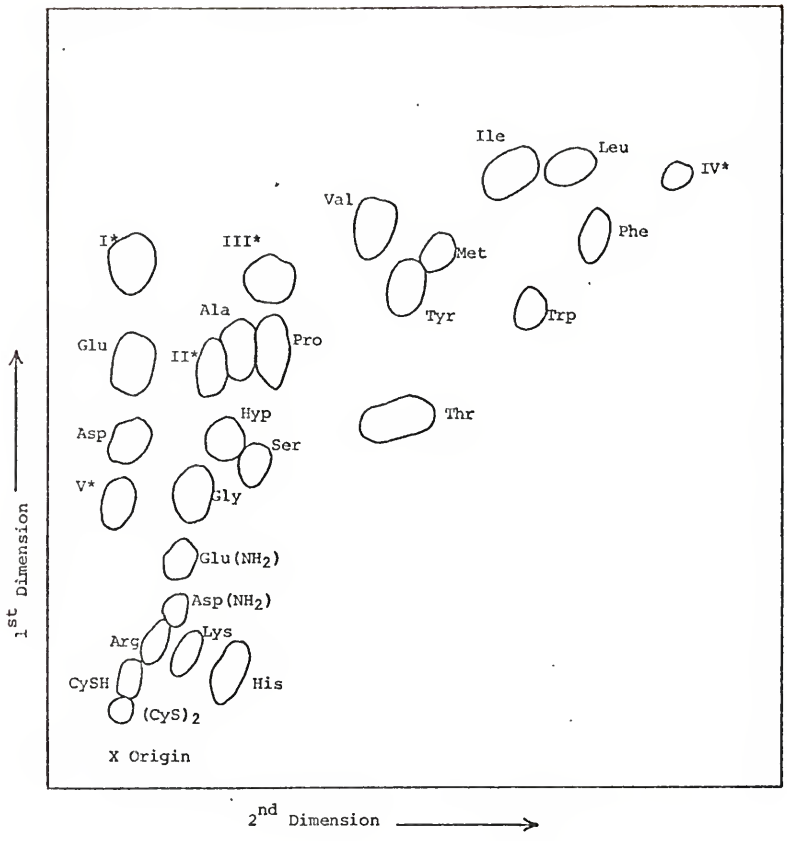

Figure 8. Separation of free amino acids extracted from sterile larval rearing medium containing ^{14}C-glucose and on which larvae were maintained.

* Unknown ninhydrin positive compounds.

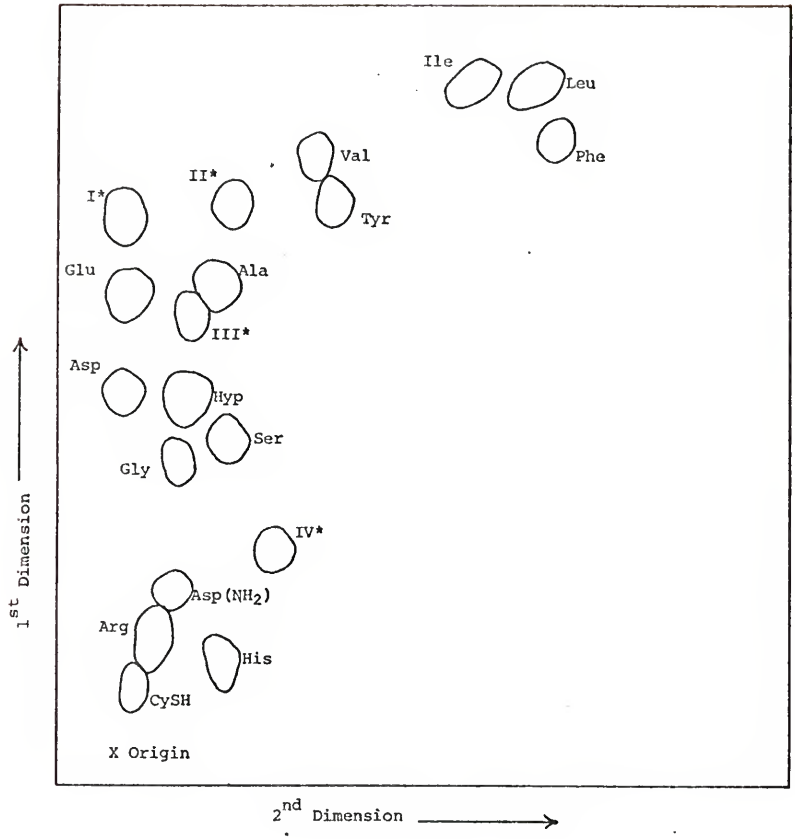

Figure 9. Separation of free amino acids extracted from non-sterile larval rearing medium containing ^{14}C-glucose and that had no larvae reared on it. (Incubated with ^{14}C-glucose for 12 weeks.)

* Unknown ninhydrin positive compounds.

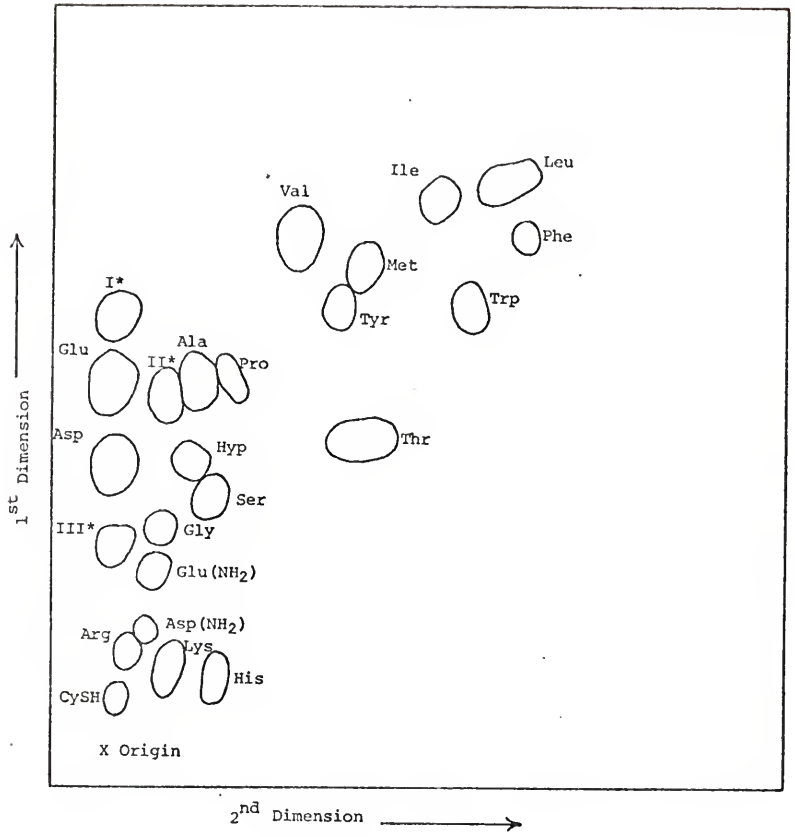

Figure 10. Separation of free amino acids extracted from sterile larval rearing medium containing ^{14}C-glucose and that had no larvae reared on it.

* Unknown ninhydrin positive compounds.

Table 6. Cpm/Carbon Atom of Free Amino Acids Extracted from Sterile Larval Rearing Medium Containing ^{14}C-Glucose and That Had Almond Moth Larvae Reared on It

Amino acid	Carbon atoms/ amino acid	Cpm+/carbon atom
Alanine	3	189.5
Arginine	6	263.6
Asparagine	4	152.7
Aspartic acid	4	223.7
Cysteine	3	1398.0
Cystine	6	162.0
Glutamic acid	5	191.5
Glutamine	5	156.3
Glycine	2	1248.0
Histidine	6	71.8
Hydroxyproline	5	169.0
Isoleucine	6	41.8
Leucine	6	46.6
Lysine	6	414.8
Methionine	5	56.5
Phenylalanine	9	18.0
Proline	5	30.0
Serine	3	113.0
Threonine	4	85.3
Tryptophan	11	5.3
Tyrosine	9	20.6
Valine	5	132.8
Unknown I*	4	213.0
Unknown II*	4	55.7
Unknown III*	4	69.0
Unknown IV*	4	54.3
Unknown V*	4	155.0

+ Cpm corrected for background and quenching.
* Cpm/carbon atom calculated on the basis of four carbon atoms per molecule.

Table 7. Cpm/Carbon Atom of Free Amino Acids Extracted from Non-Sterile Larval Rearing Medium That was Incubated with ^{14}C-Glucose for 12 Weeks and Had No Larvae Reared on It

Amino acid	Carbon atoms/ amino acid	Cpm+/carbon atom
Alanine	3	50.0
Arginine	6	41.0
Asparagine	4	24.0
Aspartic acid	4	53.0
Cysteine	3	50.0
Glutamic acid	5	32.3
Glycine	2	250.0
Histidine	6	7.0
Hydroxyproline	5	29.3
Isoleucine	6	14.2
Leucine	6	13.8
Phenylalanine	9	3.9
Serine	3	38.5
Tyrosine	9	5.9
Valine	5	33.3
Unknown I*	4	12.7
Unknown II*	4	15.3
Unknown III*	4	9.0
Unknown IV*	4	11.0

+ Cpm corrected for background and quenching.
* Cpm/carbon atom calculated on the basis of four carbon atoms per molecule.

Table 8. Cpm/Carbon Atom of Free Amino Acids Extracted from Sterile Larval Rearing Medium Containing ^{14}C-Glucose and on Which No Larvae were Reared

Amino acid	Carbon atoms/ amino acid	Cpm+/carbon atom
Alanine	3	0.0
Arginine	6	0.0
Asparagine	4	0.0
Aspartic acid	4	0.0
Cysteine	3	0.0
Glutamic acid	5	0.3
Glutamine	5	0.0
Glycine	2	0.0
Histidine	6	0.0
Hydroxyproline	5	0.0
Isoleucine	6	0.0
Leucine	6	0.0
Lysine	6	0.0
Methionine	5	0.0
Phenylalanine	9	0.0
Proline	5	0.0
Serine	3	0.0
Threonine	4	0.0
Tryptophan	11	0.0
Tyrosine	9	0.0
Valine	5	0.0
Unknown I*	4	0.0
Unknown II*	4	0.0
Unknown III*	4	0.0

+ Cpm corrected for background and quenching.
* Cpm/carbon atom calculated on the basis of four carbon atoms per molecule.

initiated the production of radioactive amino acids in the medium. This could have been accomplished either through the introduction of microorganisms into the medium by the larvae or through the excretion of radioactive amino acids into the medium by the larvae. Another possibility is that the sterile medium became contaminated from an external source. Even if this was the case, the amino acids from the sterile medium containing the larvae had much more activity than the amino acids in the non-sterile medium. This was in spite of the fact that the period of metabolism was only 4 weeks for the sterile medium containing the larvae as opposed to 12 weeks for the non-sterile medium that had no larvae on it. It is possible that the insect larvae from the surface sterilized eggs innoculated the medium with gut microorganisms which were received transovarially from the previous generation. An alternative is that the microorganisms were contained within the egg and released upon hatching of the larvae. Whatever the source of the radioactive free amino acids, and I suspect it to be microorganisms in the medium, the presence of the larvae seems to enrich the medium and enhance microbial synthesis. The other possibility is that the microbes from the insects are more efficient or adapted to the synthesis of amino acids than simple contaminating organisms. These free amino acids in the medium do not seem to contribute to the synthesis of proteins in the insect, since none of the

insect's essential amino acids that were labeled in the medium were labeled in the insect proteins. A calculation was made to determine the ratio of activity in the medium free amino acid as compared to the ^{14}C-glucose activity added to the same amount of medium (see Appendix). The activity contributed by the free amino acids to the medium at the end of the incubation period was equal to 1.4 percent of the activity contributed by the ^{14}C-glucose (see Appendix). In this low a proportion, it is difficult to see how this would help the insect meet its needs for the nutritionally essential amino acids in nature. However, with the extremely high specific activity in the cysteine, from medium free amino acids, it is possible that this could be the source of the radioactivity in the cysteine isolated from insect proteins. The free amino acids were not extracted from insect tissues to ascertain whether there was any internal synthesis of nutritionally essential free amino acids or incorporation of radioactive nutritionally essential medium free amino acids into the free amino acid pools of the insect.

In a great many studies where the continuous exposure radioactivity method was used to determine nutrient requirements and sterile technique was not used, there was synthesis of so-called essential amino acids (Kasting and McGinnis 1966). This was not the case with the present study where there was no

evidence of synthesis of essential amino acids. The almond moth larvae were reared in the presence of ^{14}C-glucose (in non-sterile medium for the first two replicates) for approximately 3 weeks and allowed to feed ad libitum. This metabolism period is approximately seven times longer than any other continuous feeding period noted in the literature. Even with this extended metabolism period, there was no incorporation of radioactive essential amino acids into insect protein. This would seem to minimize the importance of any symbiotic type of relationship, if indeed one is present, between the almond moth and any microorganism. The synthesis of nutritionally essential amino acids in the medium may be due to simple contamination of the medium with the larval presence merely enhancing the growth potential of the microbe, or true innoculation with insect associated microorganisms. This insect innoculum, if present, may be simple gut microorganisms that are insect associated but have little nutritional significance. By either route, this situation seems of little benefit to the insect with regard to essential amino acid synthesis.

A small microbiological study was undertaken to try and determine the source of the essential amino acid synthesis in the medium. Table 9 presents the results of this study which was carried out on the third replicate (i.e., sterile) of the radiometric study that was just discussed above. As can be seen

Table 9. Result of Microbiological Study on Almond Moth Eggs and Larvae and ^{14}C Media to Determine the Source of Radioactive Free Amino Acid Synthesis

Material Studied	Replicate I	Replicate II	Replicate III
Sterile medium before innoculation	-	-	-
Sterile eggs before innoculation	-	-	-
Non-sterile medium	+	+	+
Non-sterile eggs	+	+	+
Larvae from sterile eggs that had been reared on sterile medium for 1 week	+	+	+
Sterile medium that had larvae from sterile eggs reared on it for 1 week	+	+	+
2-week old sterile medium that had no larvae reared on it	-	-	-

from the table, the medium and eggs started out in an initially sterile state. Upon hatching and spending 1 week in the sterile medium, both the larvae and medium became contaminated with some type of microorganism. The control sterile medium that had no larvae reared on it was still sterile after 2 weeks. Sterile eggs placed on a number of plates hatched and the larvae burrowed into the agar. Within several days, there was microbial growth on these plates. From such a limited study, it is difficult to determine whether the microbial innoculum came from within the sterile eggs or from some form of external contamination which was enhanced by the presence of larvae on the medium. However, since there was microbial growth when larvae from sterile eggs hatched on the agar plates, it appears that the microbial innoculum came from within the almond moth eggs.

During the protein extraction procedure, the various fractions from the larval and media extractions were dried, weighed, and counted using liquid scintillation. Tables 10 through 15 present the results of these analyses. The extracts from the larval protein extraction all contain substantial activity as was expected. The cold TCA fraction contains glycogen, sugars, free amino acids, vitamins, and nucleotides. The activity in this fraction is probably contained in the glycogen which is synthesized from dietary carbohydrate (i.e., ^{14}C-glucose), the nutritionally non-essential free amino acids

Table 10. Specific Activity of Dried Supernatant Fractions from the Extraction of Fifth-Instar Almond Moth Larvae Reared on Medium Containing ^{14}C-Glucose

Supernatant fractions*	Specific activity (cpm**/mg. of extract)	Cpm**/g. of insect	% of total counts
	Replicate I		
TCA (cold)	585	173,372	9.04
Alkaline acetone	27,672	1,102,132	57.44
95% ethanol	7,456	111,699	5.82
Ether	810	2,443	0.13
TCA (hot)	379	59,391	3.10
Residue (protein)	1,852	469,852	24.49
	Replicate II		
TCA (cold)	735	196,107	10.26
Alkaline acetone	10,479	1,028,242	53.80
95% ethanol	3,852	20,775	1.09
Ether	2,015	3,073	0.16
TCA (hot)	612	42,595	2.23
Residue (protein)	3,542	620,558	32.47
	Replicate III		
TCA (cold)	504	149,667	9.37
Alkaline acetone	17,066	1,016,207	63.59
95% ethanol	3,790	78,692	4.92
Ether	996	2,512	0.16
TCA (hot)	360	40,539	2.54
Residue (protein)	1,534	310,482	19.43

* See Figure 2 for explanation of fractions.
** Cpm corrected for background and quenching.

Table 11. Specific Activity of Dried Supernatant Fractions from the Extraction of Larval Rearing Medium Containing ^{14}C-Glucose and That Had Almond Moth Larvae Reared on It

Supernatant fractions*	Specific activity (cpm**/mg. of extract)	Cpm**/g. of medium	% of total counts
	Replicate I		
TCA (cold)	5,244	1,385,239	87.63
Alkaline acetone	10,393	235,479	9.85
95% ethanol	21,781	45,851	1.97
Ether	545	310	0.02
TCA (hot)	130	11,508	0.54
	Replicate II		
TCA (cold)	7,323	1,599,430	81.72
Alkaline acetone	4,160	297,146	15.19
95% ethanol	7,196	53,416	2.73
Ether	119	237	0.02
TCA (hot)	75	6,870	0.35
	Replicate III		
TCA (cold)	6,512	1,722,520	82.53
Alkaline acetone	6,094	193,509	14.03
95% ethanol	14,095	38,751	2.73
Ether	267	289	0.02
TCA (hot)	124	10,635	0.69

* See Figure 2 for explanation of fractions.
** Cpm corrected for background and quenching.

Table 12. Specific Activity of Dried Supernatant Fractions from the Extraction of Larval Rearing Medium Containing ^{14}C-Glucose and That Had No Larvae Reared on It

Supernatant fractions*	Specific activity (cpm**/mg. of extract)	Cpm**/g. of medium	% of total counts
	Replicate I		
TCA (cold)	98	25,285	33.11
Alkaline acetone	1,319	41,496	54.35
95% ethanol	835	7,287	9.55
Ether	67	47	0.06
TCA (hot)	25	2,244	2.94
	Replicate II		
TCA (cold)	89	23,388	30.50
Alkaline acetone	795	42,172	55.01
95% ethanol	953	8,466	11.04
Ether	102	66	0.08
TCA (hot)	25	2,583	3.37
	Replicate III		
TCA (cold)	132	31,882	41.83
Alkaline acetone	768	33,221	43.59
95% ethanol	1,708	8,774	11.51
Ether	40	38	0.05
TCA (hot)	18	2,302	3.02

* See Figure 2 for explanation of fractions.
** Cpm corrected for background and quenching.

Table 13. Weights of Dried Supernatant Fractions from the Extraction of Fifth-Instar Almond Moth Larvae Reared on Medium Containing ^{14}C-Glucose

Supernatant fractions*	Weight (g.)	Mg./g. of insect extracted	% of total weight extracted
	Replicate I		
Weight of insect extracted	4.5215	-	-
TCA (cold)	1.3483	296.6	29.81
Alkaline acetone	0.3443	75.7	7.61
95% ethanol	0.1164	25.8	2.59
Ether	0.0265	5.9	0.60
TCA (hot)	0.7128	156.9	15.77
Residue weight (protein)	1.1534	253.7	25.50
	Replicate II		
Weight of insect extracted	0.8627	-	-
TCA (cold)	0.2295	266.8	26.68
Alkaline acetone	0.1436	165.9	16.59
95% ethanol	0.0076	8.8	0.88
Ether	0.0027	3.2	0.32
TCA (hot)	0.0599	69.6	6.96
Residue weight (protein)	0.1513	175.2	17.52
	Replicate III		
Weight of insect extracted	3.6124	-	-
TCA (cold)	1.0718	296.9	29.68
Alkaline acetone	0.3845	106.4	10.64
95% ethanol	0.1413	39.1	3.91
Ether	0.0177	5.0	0.50
TCA (hot)	0.4074	112.7	11.27
Residue weight (protein)	0.7225	200.3	20.02

* See Figure 2 for explanation of fractions.

Table 14. Weights of Dried Supernatant Fractions from the Extraction of Larval Rearing Medium Containing ^{14}C-Glucose and That Had Almond Moth Larvae Reared on It

Supernatant fractions*	Weight (g.)	Mg./g. of medium extracted	% of total weight extracted
	Replicate I		
Weight of medium extracted	1.0640	-	-
TCA (cold)	0.2814	264.0	26.41
Alkaline acetone	0.0517	48.6	4.86
95% ethanol	0.0083	7.7	0.77
Ether	0.0013	1.2	0.12
TCA (hot)	0.0943	88.7	8.87
Residue weight (protein)	0.3672	345.0	34.49
	Replicate II		
Weight of medium extracted	0.8241	-	-
TCA (cold)	0.1819	220.9	22.09
Alkaline acetone	0.1011	122.2	12.22
95% ethanol	0.0144	17.0	1.70
Ether	0.0041	5.1	0.51
TCA (hot)	0.0763	92.2	9.22
Residue weight (protein)	0.1670	202.4	20.27
	Replicate III		
Weight of medium extracted	1.2132	-	-
TCA (cold)	0.3211	264.5	26.47
Alkaline acetone	0.0737	60.4	6.08
95% ethanol	0.0074	6.1	0.61
Ether	0.0024	2.0	0.20
TCA (hot)	0.1043	86.0	8.60
Residue weight (protein)	0.4116	339.5	33.97

* See Figure 2 for explanation of fractions.

Table 15. Weights of Dried Supernatant Fractions from the Extraction of Larval Rearing Medium Containing ^{14}C-Glucose and That Had No Larvae Reared on It

Supernatant fractions*	Weight (g.)	Mg./g. medium extracted	% of total weight extracted
	Replicate I		
Weight of medium extracted	1.4412	-	-
TCA (cold)	0.3727	258.9	25.89
Alkaline acetone	0.0964	67.0	6.70
95% ethanol	0.0265	18.3	1.83
Ether	0.0021	1.5	0.15
TCA (hot)	0.1305	90.6	9.06
Residue weight (protein)	0.5896	409.5	40.94
	Replicate II		
Weight of medium extracted	1.5365	-	-
TCA (cold)	0.4031	262.4	26.24
Alkaline acetone	0.1529	99.6	9.96
95% ethanol	0.0287	18.7	1.87
Ether	0.0021	1.3	0.13
TCA (hot)	0.1590	103.5	10.35
Residue weight (protein)	0.4206	273.7	27.41
	Replicate III		
Weight of medium extracted	1.4820	-	-
TCA (cold)	0.3584	241.8	24.18
Alkaline acetone	0.1202	81.0	8.10
95% ethanol	0.0155	10.4	1.04
Ether	0.0028	1.9	0.19
TCA (hot)	0.1863	125.7	12.57
Residue weight (protein)	0.3173	214.0	21.39

* See Figure 2 for explanation of fractions.

which are also synthesized from ^{14}C-glucose and the purine and pyrimidine bases of the nucleotides. The pyrimidine ring contains carbon atoms that originate from CO_2 and aspartic acid. Both of these compounds are labeled via synthesis from ^{14}C-glucose. The purine ring structure contains carbon atoms that come from CO_2 and glycine. Both of these compounds are also labeled via synthesis from ^{14}C-glucose. The alkaline acetone, 95 percent ethanol, and ether fractions contain the extracted neutral and phospholipids. Dietary carbohydrate is a major precursor of lipids (Friend 1962, House 1965). A large quantity of activity would therefore be expected in the lipid extracting fractions (Table 10). This was indeed the case with approximately 83 percent of the activity from the three replicates concentrated in the three lipid extracts. The specific activity in these fractions was also high. The TCA, which was the final extracting solvent, contained nucleic acids. The activity in the nucleic acids comes from the labeling of purine and pyrimidine bases which was discussed above with respect to labeling in nucleotides.

In the extraction of protein from media that had larvae reared on them, most of the radioactivity was concentrated in the cold TCA fraction as opposed to the lipid fractions for the

larval protein extraction. Most of this activity is not simply ^{14}C-glucose being extracted off the media since the cold TCA fraction from medium that had no larvae reared on it contained only about 1/60 of the amount of activity that this medium had on it. The lipid fractions from this medium extraction did not contain a large percentage of the total counts present but they did have a very high specific activity.

The fractions from the medium that had no larvae reared on it contained small amounts of activity compared to the other medium extracted. The specific activity of these fractions was also many times lower than comparable ones from the medium that had larvae reared on it. The third replicate was carried out in duplicate for the medium that had no larvae reared on it. The sterile replicate was used only for extraction of free amino acids while the non-sterile replicate was used for all other extractions.

Several small studies related to the radiometric analysis were carried out. These are presented in the appendix.

Carcass Analysis for the Determination of Quantitative Amino Acid Requirements Using Gas Chromatography

Results of the carcass analysis of fifth-instar almond moth larvae are presented in Tables 16 and 17 and Figures 11 and 12. The gas-liquid chromatogram of 19 TMS amino acid standards along with retention time, relative retention time, and sensitivity, used to identify and quantitate the amino acids in the larvae, are presented in Table 18 and Figure 13.

Of the free amino acids in the almond moth larvae, proline, glutamic acid, and tyrosine were present in the highest concentrations making up almost 70 percent of the free amino acids present. Proline and tyrosine which participate in cuticular tanning prior to and during pupation would be expected at a high concentration in larvae this close to pupation (Chen 1962, 1966). Glutamic acid has also been reported in high concentrations among the free amino acids of insect larvae (Rock and King 1966, 1967a).

Among the protein amino acids, there was a very uniform distribution with respect to amount present. Glutamic acid and aspartic acid were the only amino acids present in substantially greater quantity than the others. This has been noted for other insects also (Rock and King 1966, 1967b, 1967c).

Table 16. Pattern of Amino Acids in Fifth-Instar Almond Moth Larvae

Amino acid	Mg. of amino acid/ g. of larvae			Mg. of amino acid/g. of protein
	Free amino acids	Protein amino acids	Total amino acids	
Alanine	.290	10.433	10.723	62.3
Valine	.150	9.134	9.284	54.5
Leucine	.224	12.864	13.088	76.8
Isoleucine	.096	8.548	8.644	51.0
Glycine	.119	5.866	5.985	35.0
Proline	2.218	6.495	8.713	38.8
Serine	.275	6.914	7.189	41.3
Threonine	.103	4.944	5.047	29.5
Hydroxyproline	-	1.215	1.215	7.3
Aspartic acid	.040	17.640	17.680	105.3
Methionine	.057	2.472	2.529	14.8
Glutamic acid	1.098	22.668	23.766	135.3
Phenylalanine	.050	6.788	6.838	40.5
Arginine	.134	6.285	6.419	37.5
Lysine	.172	10.685	10.857	63.8
Tyrosine	.531	8.590	9.121	51.3
Total	5.557	141.541	147.098	845.0

Table 17. Percent Composition of Amino Acids in Fifth-Instar Almond Moth Larvae

Amino acid	% of total free amino acids	% of total protein amino acids	% of total amino acids		
			free amino acids	protein amino acids	total amino acids
Alanine	5.22	7.37	.20	7.09	7.29
Valine	2.70	6.45	.10	6.21	6.31
Leucine	4.03	9.09	.15	8.75	8.90
Isoleucine	1.73	6.04	.07	5.81	5.88
Glycine	2.14	4.14	.08	3.99	4.07
Proline	39.91	4.59	1.51	4.42	5.93
Serine	4.95	4.89	.19	4.70	4.89
Threonine	1.85	3.49	.07	3.36	3.43
Hydroxyproline	-	.86	-	.83	.83
Aspartic acid	.72	12.46	.03	11.99	12.02
Methionine	1.03	1.75	.04	1.68	1.72
Glutamic acid	19.76	16.02	.75	15.41	16.16
Phenylalanine	.90	4.80	.03	4.62	4.65
Arginine	2.41	4.44	.09	4.27	4.36
Lysine	3.10	7.55	.12	7.26	7.38
Tyrosine	9.56	6.07	.36	5.84	6.20
Total	100.01	100.01	3.79	96.23	100.02

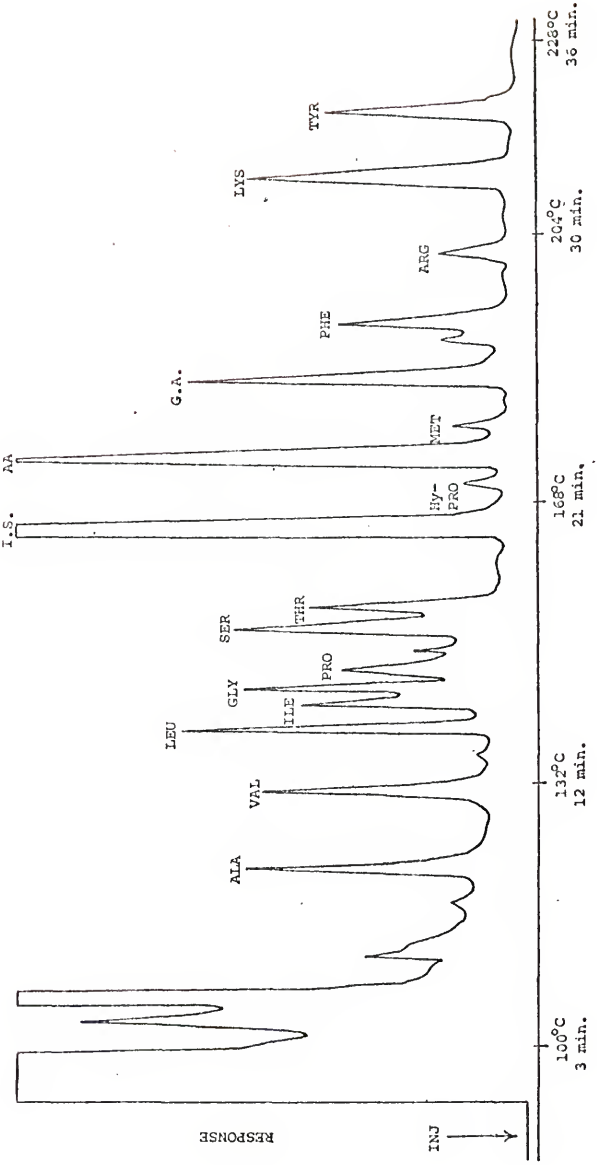

Figure 11. Gas-liquid chromatogram of TMS amino acid derivatives from protein hydrolysate of fifth-instar almond moth larvae. Sample: 4 mg.

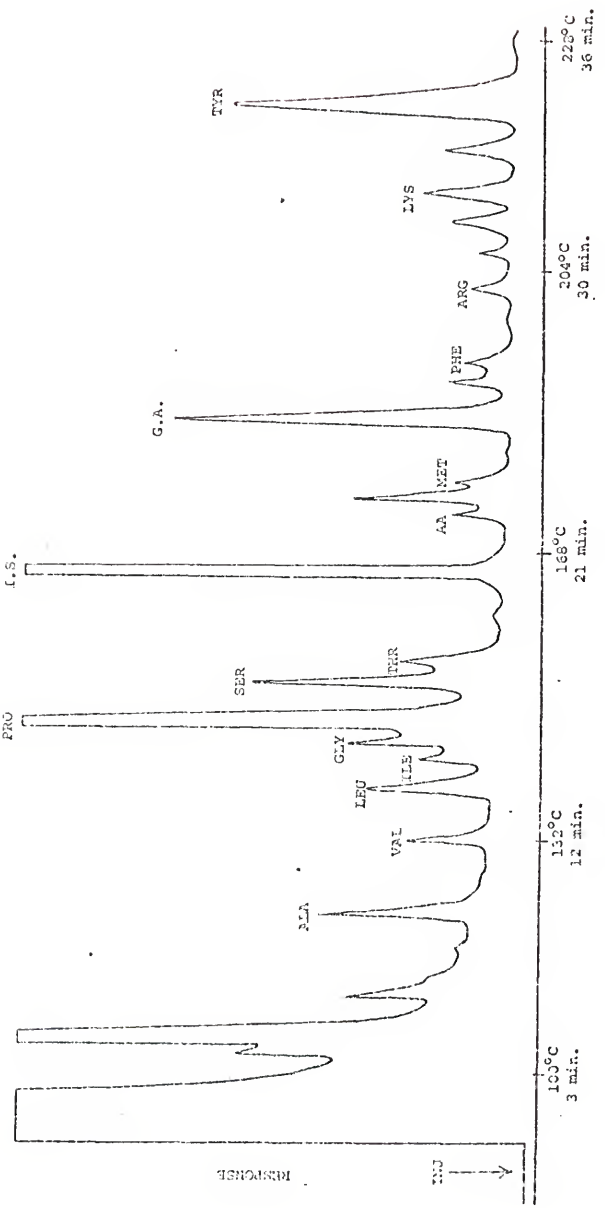

Figure 12. Gas-liquid chromatogram of TMS-free amino acids extracted from fifth-instar almond moth larvae. Sample was cation-exchange cleaned.

Table 18. Retention Time, Relative Retention Time, and Sensitivity of 19 Trimethylsilyl Amino Acid Standards

Amino Acid	Retention Time (sec.)	Relative Retention Time	Sensitivity (ng./mm. of recorder deflection)
Alanine	.525	.432	11.6
Valine	725	.597	10.4
Leucine	825	.679	10.9
Isoleucine	870	.716	21.7
Glycine	895	.737	9.3
Proline	930	.765	16.1
Serine	1000	.823	8.5
Threonine	1035	.852	9.6
Internal Standard (Decanoic acid)	1215	1.000	-
Hydroxyproline	1300	1.070	16.1
Aspartic acid	1320	1.086	9.4
Methionine	1370	1.128	17.2
Cysteine	1400	1.152	35.7
Glutamic acid	1475	1.214	18.5
Phenylalanine	1565	1.288	12.2
Arginine	1735	1.428	22.7
Lysine	1890	1.556	11.4
Tyrosine	2020	1.663	11.9
Tryptophan	2500	2.058	26.3
Cystine	2520	2.074	71.4

NOTE: Standard column and conditions - Column: 10 percent OV-11 on Supelcoport 100/120 mesh. 12 ft. x 2 mm. I.D. Conditions: injector temperature 275°C.; detector temperature, 300°C.; oven temperature, initial 100°C.; 3-min. hold after start of solvent peak, 4°C./min. increased to 300°C.; attenuator settings 32 x 10^{-11}; N_2 carrier gas flow, 17 ml./min.; flame ionization detector; internal standard, Decanoic acid.

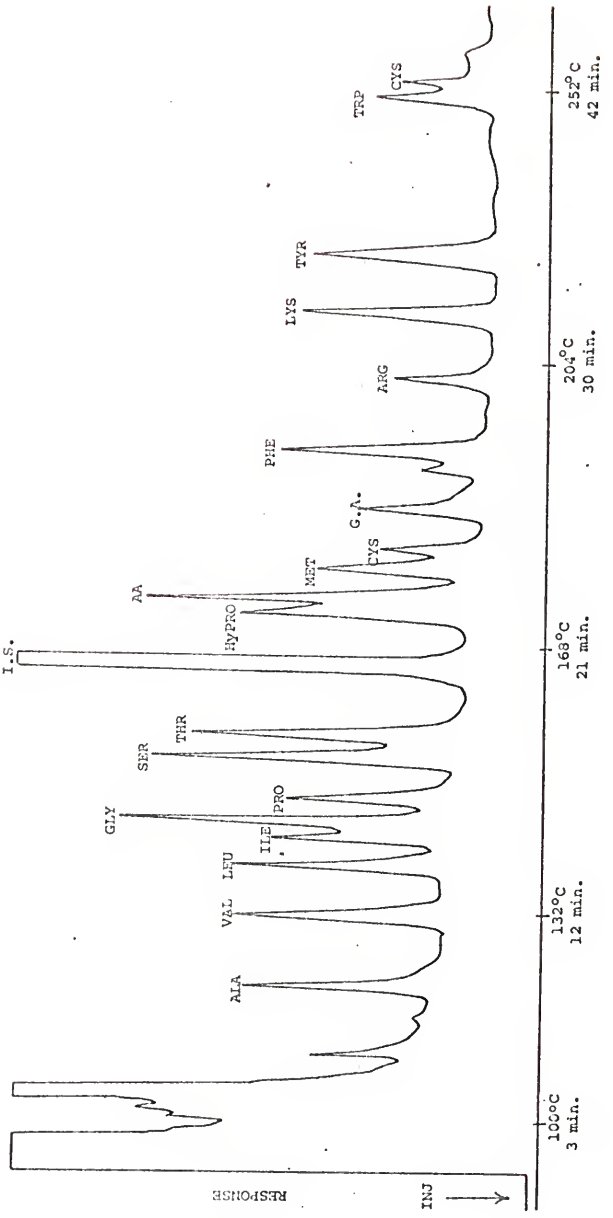

Figure 13. Gas-liquid chromatogram of TMS protein amino acid standards. Sample: 2.0 mg. in 1.0 ml. 0.5 µg each amino acid injected. BSTFA-CH_3CN (1:1), Closed tube silylation at 150°C. for 2.5 hr. Column: 10 percent OV-11 on Supelcoport 100/120 mesh. 12 ft. x 2 mm. I.D. Conditions: injector, 275°C.; detector, 300°C.; oven, initial 100°C.; 3 min. hold after start of solvent peak, 4°C./min. increase to 300°C.; carrier gas N_2, 17 ml./min. I.S. = Decanoic acid.

A small amount of hydroxyproline was detected among the protein amino acids. This is very unusual since hydroxyproline is not commonly found in proteins. It has, however, been found in fibrous proteins, such as collagen, and in plant proteins.

Table 19 is a proposed mixture, at the 2 percent and 3 percent dietary level, of amino acids based on results from carcass analysis of fifth-instar almond moth larvae. Tryptophan is not included in this table since it is destroyed by acid hydrolysis and no base hydrolysates were analyzed. Histidine, cysteine, and cystine were not detected with the GLC method as they were with TLC. Cysteine and cystine are often in very low concentrations in late instar lepidoptera larvae (Rock and King 1966, 1967c).

A study was done to determine if there was any loss of amino acids during the protein extraction procedures. Figure 14 presents the results of this study. The derivatized extraction fractions contained no significant chromatographic peaks, aside from the internal standard, both before and after acid hydrolysis. This is not surprising since amino acids and peptides are markedly hydrophilic and thus very insoluble in non-aqueous solvents. The organic solvents used for the extraction procedure (i.e., alkaline acetone, ether, and 95 percent ethanol) would have very little affinity for amino acids and not contribute to the loss of these compounds. The aqueous

Table 19. Amino Acid Mixture Patterned After Carcass Analysis of Fifth-Instar Almond Moth Larvae

Amino Acid	2% Dietary level (mg./100 g. of diet)	3% Dietary level (mg./100 g. of diet)	Dietary requirement from ^{14}C study
Alanine	146	219	-
Valine	126	189	+
Leucine	178	267	+
Isoleucine	118	177	+
Glycine	82	123	-
Proline	118	177	-
Serine	98	147	-
Threonine	68	102	+
Aspartic acid	240	360	-
Methionine	34	51	+
Glutamic acid	324	486	-
Phenylalanine	94	141	+
Arginine	88	132	+
Lysine	148	222	+
Tyrosine	124	186	+
Hydroxyproline	16	24	

+ = Not synthesized - nutritionally essential
- = Synthesized - nutritionally non-essential.

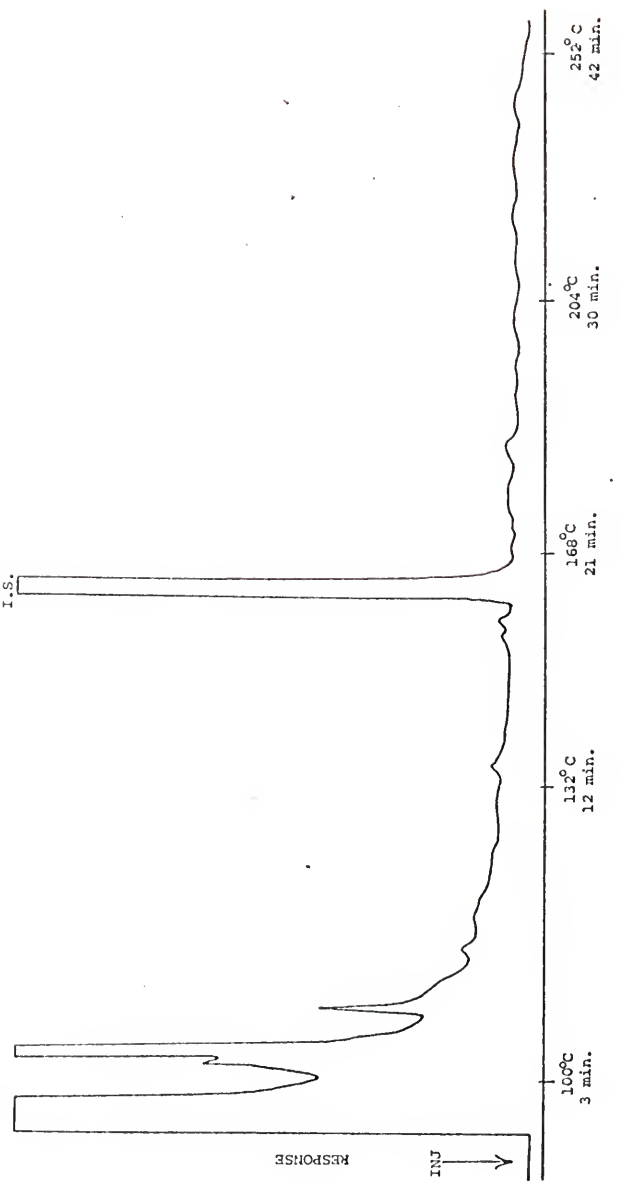

Figure 14. Gas-liquid chromatogram of a TMS derivatization of an alkaline acetone fraction from the extraction of protein from almond moth larvae. The extract was evaporated to dryness and hydrolyzed before derivatization. The chromatograms of the 95 percent ethanol fraction, ether fraction, and hot TCA fraction, both before and after acid hydrolysis, appeared the same.

cold TCA fraction takes out the free amino acids from the insect tissue in the first extraction step and it was this fraction that was analyzed for free amino acids of the insect tissues.

The almond moth required the same 10 amino acids as did the eight insects in Table 1, in addition to tryptophan. It did not synthesize or require any amino acid that had not been reported previously for another insect.

The percentage composition of amino acids in the almond moth was quite similar to that found in other insects (Rock and King 1966, 1967b, 1967c). Free amino acids consisted mainly of proline, glutamic acid, and tyrosine (Chen 1962, 1966, Rock and King 1966, 1967a) while, among the protein amino acids, glutamic acid and aspartic acid predominated with the others being present in fairly equal concentration (Rock and King 1966, 1967b, 1967c).

The presence of some type of insect-microorganism relationship in the almond moth has not been noted previously. House (1962) notes that symbiotes usually occur in species that feed exclusively on substances such as plant sap, vertebrate blood, and certain stored products that are deficient in specific nutrients. Fraenkel (1959), on the other hand, states that the need by many stored product insects for eight or nine B-complex

vitamins, even when reared in the absence of axenic conditions, minimizes the nutritional importance of microorganisms that are, no doubt, present in the intestine. He also states that due to the dry, powdery nature of the diet of stored product insects, microbial actions in the medium would be almost non-existent. In the almond moth medium, there is substantial microbial action, as evidenced by the large number of substantially labeled free amino acids isolated here. However, the nutritional importance of this action seems at present of only slight value. With the possible exception of cysteine, there was no incorporation of labeled essential medium free amino acids into the protein of the larval almond moth. With the continuous exposure method of isotope administration, there is often microbial synthesis of essential free amino acids, with subsequent incorporation into the insect larvae (Kasting and McGinnis 1966). This was not the case with the almond moth, even though it was exposed to the ^{14}C-glucose for a much longer time period than in most continuous exposure nutritional studies.

With the incorporation of the data from the radiometric study with those obtained from the carcass analysis study, the dietary amino acid requirements of the almond moth have been identified. These data have also been used to formulate an amino acid ration which should be an excellent starting point for formulating a

completely defined diet for the almond moth. Once the almond moth can be reared on a completely defined diet, nutritional state can be eliminated as a variable in any future studies with this insect pest.

SUMMARY

The almond moth synthesizes alanine, aspartic acid, glutamic acid, glycine, proline, and serine from ^{14}C-glucose. These amino acids are considered nutritionally non-essential. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine were not synthesized and are considered nutritionally essential. Cysteine and cystine were synthesized to a limited degree and cannot yet be classified.

Radioactive essential and non-essential free amino acids were isolated from larval rearing media. The specific activities were substantially higher in amino acids extracted from medium that had larvae reared on it than from medium that had no larvae reared on it. There appears to be an insect-microorganism relationship at work here. However, the exact nature of this relationship and to what extent it contributes to the insects' nutrition are at present undetermined. This situation seems to contradict what some authors have stated that, due to the dry nature of the stored product insects' diet, microbial associations or contamination should not be a factor (Fraenkel 1959). However, since there was no detectable incorporation of radioactive essential free amino acids from the medium into the larval proteins (with the possible exception of cysteine), the

nutritional importance of this relationship seems questionable when the techniques I employed in this study are used.

In other radiometric nutrition studies with insects, where the continuous exposure method of isotope incorporation was used, there appeared to be synthesis of essential amino acids by the insect (Kasting and McGinnis 1966). This synthesis was presumably microbial in nature. Even though the period of exposure to the ^{14}C-glucose was approximately seven times longer in my study than with other continuous exposure studies, there was still no appreciable synthesis of essential amino acids by the larvae or any related microorganisms. This factor in addition to the one stated previously tends to minimize the nutritional importance of the insect-microorganism relationship with the techniques employed in this study.

The study to isolate the source of the insect associated microorganisms is at present inconclusive. However, from the data available presently, it appears that the microorganisms come from within the almond moth eggs.

From carcass analysis of fifth-instar almond moth larvae, it was determined that proline, tyrosine, and glutamic acid make up almost 70 percent of the total free amino acids present. Proline and tyrosine participate in cuticular tanning at this time just prior to pupation. The protein amino acids, which contain a high percentage of glutamic acid and aspartic acid, in combination with the free amino acids are the basis for a dietary mixture at the 2 percent and 3 percent levels (Table 19).

APPENDIX

The results of a number of small radiometric studies are presented here in the appendix.

Before a tracer study can be carried out, the purity of the labeled substrate must be ascertained. This was accomplished by one-dimensional thin-layer co-chromatography of the ^{14}C-glucose with a non-radioactive glucose standard followed by autoradiography of the TLC plate. Figure A-1 presents the results of this study. As can be seen, there is a single dark spot on the x-ray film which corresponds to the position of glucose on the TLC plate. This single spot on the film demonstrates that there are no other detectable radioactive substrates present which could be metabolized by the larvae and invalidate the results.

There are compounds that when placed in a liquid scintillation counter excite the cocktail or photomultiplier tube causing counts to be recorded when none in fact are present. The thin-layer adsorbent and ninhydrin dye were therefore checked to see if they caused the production of spurious counts. Table A-1 presents the results of this study. For the samples of adsorbent and dye counted, both alone and in combination, there was no increase in counts upon the addition of these substances to the scintillation vials in various amounts. It was therefore concluded that these two substances would not add to the activity of samples counted from the TLC plates.

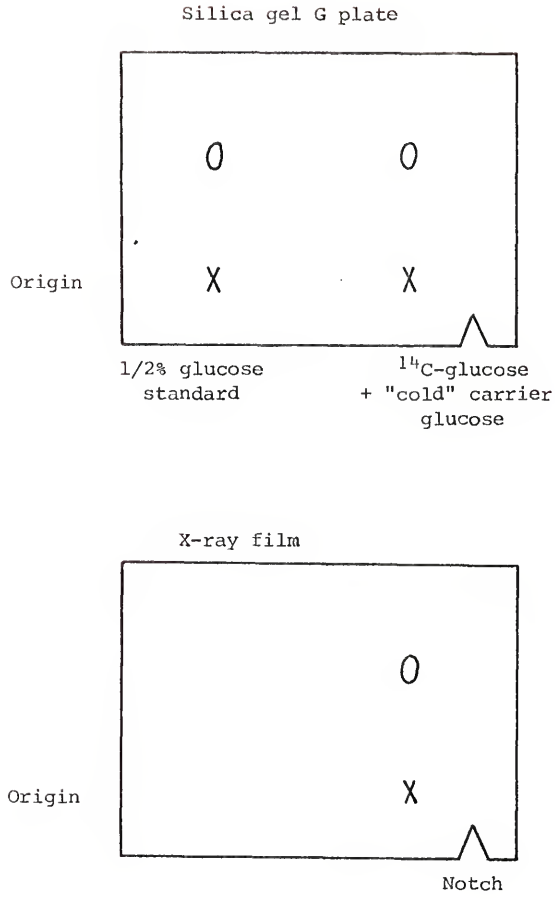

Figure A-1. One-dimensional co-chromatography of ^{14}C-glucose with 1/2% glucose standard (in 3% aqueous ethanol) followed by autoradiography of the thin-layer plate on Kodak No-Screen X-ray Film.

Table A-1. Effect of Thin-Layer Adsorbent and Thin-Layer Adsorbent and Ninhydrin in Combination on Counting System Background

Components added to scintillation vial*	Cpm before addition of cellulose adsorbent + ninhydrin	Cpm after addition of cellulose adsorbent + ninhydrin
1 visualized amino acid spot area**	32	35
2 visualized amino acid spot areas	36	35
4 visualized amino acid spot areas	34	34
8 visualized amino acid spot areas	36	34
1 non-visualized amino acid spot area	36	37
2 non-visualized amino acid spot areas	35	37
4 non-visualized amino acid spot areas	36	37
8 non-visualized amino acid spot areas	37	36

* Cocktail = 5 g. PPO, 0.3 g. POPOP, 1 L. of toluene and Cab-O-Sil (4% w/w). 15 ml./vial.

** Spot size approximately 1.5-2 cm. in diameter.

The quenching that was caused during scintillation counting by amino acids from thin-layer plates and the protein extraction fractions was corrected for by automatic external standardization (AES). A study to determine the accuracy of this method by comparing it to the internal standardization method was carried out (see Tables A-2, A-3, and A-4). The amount of quenching caused by 0.2 mg. of undyed amino acid is quite small, ranging from 1.4 percent to 7.7 percent for the 20 amino acids tested. The same quantity of amino acids stained with ninhydrin caused considerably more quenching, ranging from 7.7 percent to 28.8 percent, for the various amino acids tested. If the quenching caused by the gel and cellulose plate blank, which measured substantially more with the AES method (20 percent versus 1.3 percent), is subtracted from the quenching values obtained with the two methods, the values are reasonably close in most cases. The internal standard values are on the average higher than the AES values which were obtained from the actual experimental samples. The low quenching values obtained for cysteine, cystine, proline, and hydroxyproline with both methods, are consistent with the low color yield obtained from these amino acids with the ninhydrin reaction (Alexander and Block 1960b).

Table A-2. Quenching Properties of 20 Non-Visualized Amino Acid Standards Adsorbed on Cellulose Thin-Layers Using the Internal Standardization Method

Sample	Cpm before addition of amino acid	Cpm after addition of amino acid	Difference	Percent Quenching
Gel blank	5117	5040	-77	1.5
Gel + cellulose plate blank	3337	3290	-47	1.4
Aspartic acid	3987	3790	-197	4.9
Arginine	4440	4382	-58	1.3
Alanine	4927	4714	-213	4.3
Cysteine	2304	2251	-53	2.3
Cystine	4917	4847	-70	1.4
Glutamic acid	4891	4783	-108	2.2
Glycine	4154	3838	-306	7.4
Histidine	5107	4976	-131	2.6
Hydroxyproline	5210	4954	-256	4.9
Isoleucine	5096	4859	-237	4.7
Leucine	5100	4743	-357	7.0
Lysine	5195	4799	-396	7.6
Methionine	5237	4834	-403	7.7
Phenylalanine	5200	4830	-370	7.1
Proline	5196	4918	-278	5.4
Serine	5275	4902	-373	7.1
Threonine	4008	3762	-246	6.1
Tryptophan	5234	4951	-283	5.4
Tyrosine	5250	4866	-384	7.3
Valine	5181	4888	-293	5.7
Blank	5144	5153	-	-

NOTE: 0.2 mg. of amino acid standard was added to each scintillation vial.

Table A-3. Quenching Properties of 20 Amino Acid Standards Adsorbed on Cellulose Thin-Layers and Visualized with 1/2 Percent Ninhydrin in Acetone Spray Using the Internal Standardization Method

Sample	Cpm before addition of amino acid	Cpm after addition of amino acid	Difference	Percent Quenching
Gel blank	2468	2441	-27	1.1
Gel + cellulose plate blank	2614	2583	-31	1.2
Aspartic acid	2314	1881	-433	18.7
Alanine	2866	2377	-489	17.1
Arginine	2994	2679	-315	10.5
Cysteine	1268	1162	-106	8.4
Cystine	2841	2623	-218	7.7
Glutamic acid	2863	2477	-386	13.5
Glycine	2308	1768	-540	23.4
Histidine	3016	2660	-356	11.8
Hydroxyproline	2896	2634	-262	9.0
Isoleucine	2905	2363	-542	18.7
Leucine	2713	1933	-780	28.8
Lysine	2725	2122	-603	22.1
Methionine	2786	2301	-485	17.4
Phenylalanine	2802	2406	-396	14.1
Proline	2973	2711	-262	8.8
Serine	2917	2605	-312	10.7
Threonine	2220	1890	-330	14.9
Tryptophan	2931	2581	-350	11.9
Tyrosine	2868	2511	-357	12.4
Valine	2863	2275	-588	20.5
Blank	1986	1992	-	-

NOTE: 0.2 mg. of visualized amino acid standard was added to each scintillation vial.

Table A-4. Quenching Properties of Visualized Amino Acids from Larval Protein Hydrolysates Separated by TLC and Using Automatic External Standardization

Amino acid	Mg. of amino acid	Percent Quenching*
Alanine	0.125	31
Arginine	0.075	19
Aspartic acid	0.211	27
Cysteine	-	28
Cystine	-	20
Glutamic acid	0.271	31
Glycine	0.070	26
Histidine	-	26
Isoleucine	0.102	42
Leucine	0.154	50
Lysine	0.128	35
Methionine	0.030	22
Phenylalanine	0.081	29
Proline	0.078	21
Serine	0.083	25
Threonine	0.059	32
Tryptophan	-	25
Tyrosine	0.103	26
Valine	0.109	39
Gel blank	-	20

* Mean of three replicates.

The quenching properties of dried supernatant fractions from the TCA extraction of protein from almond moth larvae were examined next (see Tables A-5 and A-6). The high quenching values obtained with both methods are not surprising considering that many of the extracts contained large amounts of solid material and were either quite colored or cloudy in appearance. Again with the AES method, the quenching measurement for the background cocktail was considerably higher (i.e., approximately 3 times higher) than that measured by the internal standard method. Following subtraction of background quenching from the samples, the average of the three AES method replicates was compared with the internal standard method. The results of both methods are in fairly good agreement, with only the hot TCA and ether fractions being out of line.

Table A-5. Quenching Properties of Five Supernatant Fractions from the Extraction of Larval Proteins Using the Internal Standardization Method

Fraction	Amount of extract added* (mg.)	Cpm before addition of extract	Cpm after addition of extract	Difference	Percent Quenching
Blank	-	5144	5153	-	-
Soluene blank	-	5161	4738	-423	8.2
TCA** I	624	5209	2059	-3150	60.5
Alkaline acetone I	146	5101	2523	-2578	51.5
95% Ethanol I	55	5149	3219	-1930	37.5
Ether I	7	5220	3860	-1360	26.1
TCA II	266	5204	2787	-2417	46.5

* Fractions added were from the extraction of non-labeled proteins.

** Trichloroacetic acid.

Table A-6. Quenching Properties of Supernatant Fractions from the Extraction of Larval Protein Using Automatic External Standardization

Supernatant fraction	Weight of fraction* (mg.)	Percent Quenching*
Soluene blank	-	23.3
TCA I	883.3	59.3
Alkaline acetone I	208.7	59.7
95% ethanol I	69.3	47.7
Ether I	9.0	28.0
TCA II	393.3	61.7

* Mean of three replicates.

RATIO OF RADIOACTIVITY IN FREE AMINO ACIDS EXTRACTED FROM STERILE LARVAL REARING MEDIUM THAT HAD LARVAE REARED ON IT TO RADIOACTIVITY IN ^{14}C-GLUCOSE ADDED TO THE SAME MEDIUM

Total medium made radioactive = 30 g. = 30,000 mg.

Amount of medium extracted for free amino acids = .65 g. = 650 mg.

^{14}C-glucose added to jar of medium = 160 µCi.

160 uCi. = 3.52×10^8 dpm/jar of medium.

Diet mixed well - approximately uniform distribution of ^{14}C-glucose.

Dpm/mg. diet = 11,733 from glucose.

Dpm from ^{14}C-glucose in 650 mg. of diet extracted for free amino acids = 7, 626, 450.

Cpm from free amino acids in 650 mg. of medium (added counts from ninhydrin positive spots, both known and unknown).

Total volume of extract after concentration = 250 µl.

5 µl spotted per TLC plate.

10 TLC plates combined for each compound counted = 50 µl.

Counts* in 50 µl of free amino acid extract = 15,984 cpm.

Counts* in 250 µl of free amino acid extract = 79,920 cpm.

Machine efficiency approximately 75 percent. Therefore, 79,920 cpm = 106,560 dpm from free amino acids in total extract.

$\frac{106,560}{7,626,450}$ dpm from total radioactive free amino acids in 650 mg. of medium.
dpm from total ^{14}C-glucose in 650 mg. of medium.

Therefore, dpm from labeled free amino acids in medium approximately 1.4 percent of dpm from ^{14}C-glucose in medium.

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* Cpm corrected for background and quenching.

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BIOGRAPHICAL SKETCH

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Jack Myron Heller is married to the former Barbara Elaine Firstenberg and has two children, Brett Michael, age 5, and Michelle Lisa, age 3.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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